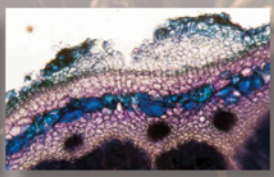
Takuji Ohyama et al.





Nitrogen Fixation *and* Metabolism *in* Soybean Plants

Novinka

NITROGEN FIXATION AND METABOLISM IN SOYBEAN PLANTS

NITROGEN FIXATION AND METABOLISM IN SOYBEAN PLANTS

TAKUJI OHYAMA, NORIKUNI OHTAKE,
KUNI SUEYOSHI, KAUSHAL TEWARI,
YOSHIHIKO TAKAHASHI, SAYURI ITO,
TOSHIKAZU NISHIWAKI,
YOSHIFUMI NAGUMO, SATOMI ISHII
AND
TAKASHI SATO

Nova Science Publishers, Inc.
New York

LIBRARY OF CONGRESS CATALOGING-IN-PUBLICATION DATA

ISBN: 978-1-60741-277-9 (E-Book)

Available upon request

Published by Nova Science Publishers, Inc. † New York

CONTENTS

PREFACE

In the first part, we would like to introduce the progress of researches on nitrogen metabolism of soybean nodules and roots. We investigated the fate of nitrogen fixed in soybean nodules by tracer experiment with $^{15}N_2$ gas. The results indicated that major part of fixed N in bacteroids (a symbiotic state of rhizobia) is excreted rapidly to cytosol of infected cells in the form of ammonia, then the ammonia is assimilated into amino acids via GS/GOGAT pathway. Then the fixed nitrogen is assimilated into ureides, allantoin and allantoic acid, and then transported to the shoots via xylem. A small portion of fixed N was assimilated in the bacteroids directly into glutamate and alanine. On the other hand, nitrate absorbed from the roots are mainly assimilated into asparagine. The characteristics of nitrate absorption and metabolism was studied.

It is well known that nitrate is a potent inhibitor to nodulation and nitrogen fixation, although the inhibitory mechanism is not fully understood. We recently found that nitrate depresses individual nodule growth and nitrogen fixation activity rapidly and reversibly when nodules were in direct contact with nitrate. The indirect effects of nitrate on nodule growth and nitrogen fixation activity were different among treatment concentration and period of supply. The continuous supply of low levels of nitrate from the lower part of roots promoted the nodulation and nitrogen fixation of the upper part of the roots. Hypernodulation mutant lines of soybean were isolated which have profuse nodulation compared with parents. They also exhibit partial-nitrate tolerant to nodulation. The characteristics of hypernodulation mutant lines were studied in relation to nitrate inhibition. The results suggested that lower nitrate absorption and assimilation activity in hypernodulation mutants may be one reason to milder inhibition by nitrate on hypernodulation mutant lines.

Soybean seed contains two major storage protein, glycinin and β-conglycinin. We discovered that nitrogen nutrition affects seed storage protein subunit accumulation. When soybean plants suffer N deficiency, soybean seed did not accumulate the β-subunit of β-conglycinin.

The new fertilization technique, deep placement of slow release nitrogen fertilizer, which promote nitrogen fixation and seed yield of soybean. Slow release N fertilizer (coated urea) of 100 kg N ha^{-1} was applied at 20cm below soil surface. The N released from the deep place did not inhibit nodulation and nitrogen fixation activity, then promoted total N accumulation and seed yield. Also we used lime nitrogen fertilizer which contain calcium cyanamide is as effective as coated urea fertilizer.

Chapter 1

INTRODUCTION

1. IMPORTANCE OF NITROGEN FIXATION IN SOYBEAN CULTIVATION

Leguminous plant consists a large group about 18,000 species including annual grasses and perennial trees. The origin of legume was dated at about 59 million years before present, with three subfamilies, Caesalpinioideae, Mimosoideae, and Papilionoideae recognized soon after (Sprent and James 2007). Although only small numbers of leguminous species are selected as crops, they are very important for foods and feed for animals world wide. Soybean (*Glycine max* (L.) Merr.) seed (Figure 1) production is 215 million t $year^{-1}$ in 2005 (FAOSTAT), accounts for a half of all the leguminous crops due to the nutritional value both for human and livestocks. Major soybean production countries (annual production million t $year^{-1}$ in 2005), are USA (85), Brazil (51), Argentina (38), China (17), and India (7). The world average seed yield is 2.3 t ha^{-1} in 2005. Soybean production in Japan was 225,000 t, and the seed yield was 1.68 t ha^{-1} in 2005.

The highest yield of soybean in Japan was recorded at 7.8 t ha^{-1} (Konno 1976), and soybean seed yield can reach 4-6 t ha^{-1} with well-managed fields under good climatic and soil conditions (e.g. Takahashi et al. 1992). The low average yield compared with potential productivity may be due to several reasons. First, soybean plants are very susceptible to physical, chemical and biological conditions of soil as well as climatic conditions. Figure 2 shows an example of giant soybean cv. Williams cultivated with low planting density at 2 plants m^{-2} (Suganuma et al. 2001). This plant had very thick basal part of stem (25 mm

diameter) and it had 1874 nodules on the roots, and 600 pods as shown in Figure 3, in which all the leaves are removed to show structures of stems. Second, soybean seed yield often severely declines with pest infection, insects, and weeds. Third, nitrogen fixation with rhizobia is very important for soybean production (Atkins 1986, Bohlool et al. 1992, Keyser and Li, 1992), but it is difficult to obtain optimum condition of nitrogen fixation. It is well known that soybean plants can fix atmospheric N_2 by the root nodule (Figure 4), which is a symbiotic organ with soil bacteria, rhizobia. However, the nodule formation and nitrogen fixation is very sensitive to deleterious environmental conditions. Therefore, many stress conditions, such as a shortage of water, decrease in oxygen supply in soil, high or low pH, nutrient deficiency or imbalance etc. may depress nodule formation and nitrogen fixation activity. In addition, low population of compatible rhizobia or the dominance of inefficient strains of indigenous rhizobia in the field may decrease nitrogen fixation activity.

Figure 1. Photograph of seeds of soybean (*Glycine max* [L.] Merr), cultivar Enrei.

In Niigata fields, about 60-75% of N assimilation in soybean was shown to derive from N_2 fixation (Takahashi et al. 1993a, Ohyama et al. 1992). Figure 5 shows nodulated soybean plants cv. Enrei and the non-nodulated isogenic line En1282 planted in the field of Niigata Agricultural Research Institute, Nagaoka. It is obvious that non-nodulated soybean grew very poor with pale leaf color due to N deficiency by the lack of nitrogen fixation. The legume nitrogen fixation is

variable, but it is a valuable process in world agriculture (Harderson 1993, Herridge and Danso, 1995, Herridge and Rose 2000)

Figure 2. One plant of soybean cv. Williams cultivated with low density 2 plants m^{-2}. (From Suganuma et al. 2001)

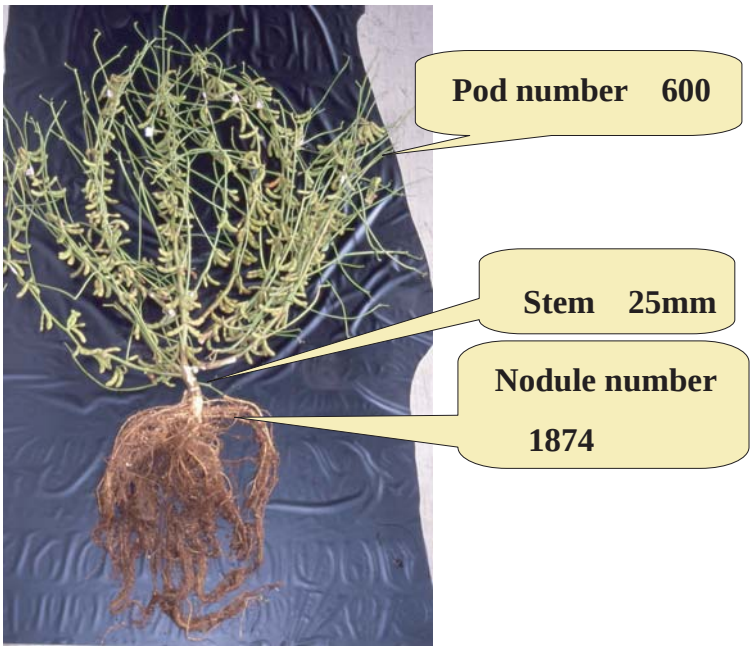


Figure 3. Soybean cv. Williams cultivated with low density 2 plants m^{-2}. All the leaves were removed.(From Suganuma et al. 2001)

Figure 4. Photograph of nodulated root of soybean (cv. Williams).

Figure 5. Comparison of nodulated (left) soybean (cv. Enrei) and the non-nodulated isoline (En1282) grown in the same field.

Soybean seeds contain a large amount of protein N and the total amount of N assimilated in a plant is highly correlated with the soybean seed yield. One t of

soybean seed requires about 70-90 kg N, which is about four times more than in the case of rice (Hoshi 1982). Soybean plants assimilate the N from three sources, N derived from atmospheric nitrogen by symbiotic N_2 fixation in root nodules (Ndfa), absorbed N derived from soil mineralized N (Ndfs), and N derived from fertilizer when applied (Ndff) (Figure 6). For the maximum seed yield of soybean, it is necessary to use both N_2 fixation and absorbed N from roots (Harper 1974, 1987). Sole N_2 fixation is often insufficient to support vigorous vegetative growth, which results in the reduction of seed yield. On the other hand, a heavy supply of N fertilizer often depresses nodule development and N_2 fixation activity and induces nodule senescence, which sometimes results in the reduction of seed yield. In addition N fertilizer often causes over luxuriant growth, which resulted in lodging or poor pod formation. Therefore, no nitrogen fertilizer is applied for soybean cultivation or only a small amount of N fertilizer is applied as a starter N to promote initial growth.

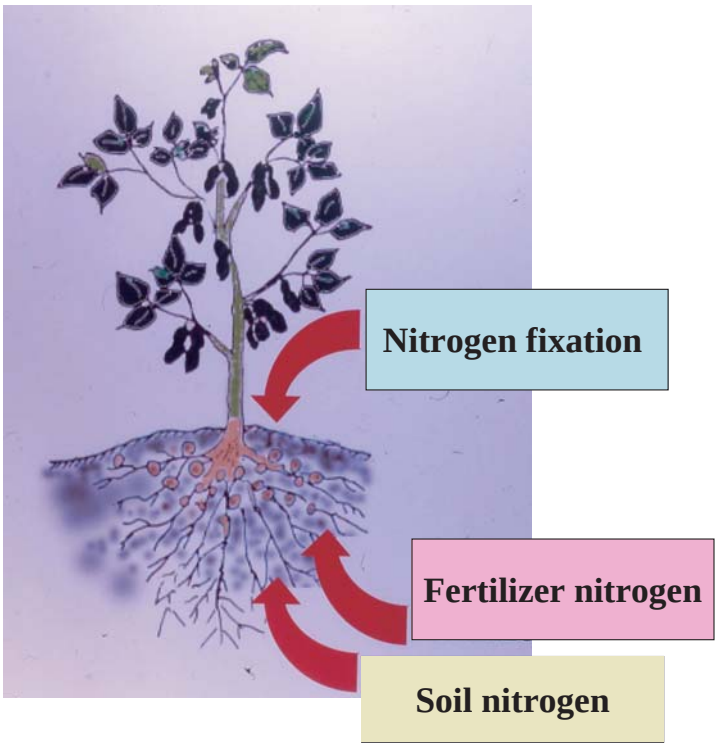


Figure 6. Three sources of N for soybean growth

2. RECENT ADVANCES IN INFECTION AND NODULATION PROCESSES

Recently, the recognition, host specificity and initial nodulation processes between leguminous plants and compatible rhizobia has been rapidly uncovered. There are many good reviews about this aspect (Rolfe and Gresshoff 1988, Sprent 1989, Denarie et al. 1992, Hirsch and Fang 1994, Spaink 1995, Stacey 1995, Crespi and Galves 2000, Lhuissier et al. 2001, Stougaard J, 2001, Lum and Hirsch 2003, Ferguson and Mathesius 2003, Limpens and Bisseling, 2003), so we briefly introduce the outline shown in Figure 7.

1). Rhizobia live heterotrophically depending on the organic matter in soil, and they don't fix atmospheric N_2. The host legume roots excrete species specific isoflavonoid compounds. Daizein and genistein are two major isoflavonoids released from soybean roots.
2). Compatible rhizobia, usually species *Bradyrhizobium japonicum* for soybean, recognize the isoflavonoid released from host legume, and NOD genes are expressed by specific isoflavonoid signals to make NOD factor. The NOD factor is a lipochitine oligosaccharide with some modification. The structures of NOD factors are different among rhizobia species and only compatible NOD factor can induce nodule formation in host plants with very low concentrations. The cell division in the cortex restores to prepare nodule formation.
3). Rhizobia move to the host roots and proliferate near the root surface.
4). Rhizobia attached to the extending root hair.
5). Then root hair entraps the rhizobia by root hair curling. Host plant makes the infection thread, which has a tunnel like structure, and rhizobia can enter into the roots through it. Finally rhizobia are released into the proliferating nodule meristem cells. One or several rhizobia is enclosed in PBM (peribacteroid membrane) or symbiosome membrane in synonym.
6). Plant cell division and rhizobium proliferation occur and nodule structure develops.
7). Nodule vascular bundles connect to the root vascular bundles and nodules and roots exchange materials through phloem and xylem. Nodules are formed and bacteroid, a symbiotic state of rhizobia, start to fix N_2. In the case of soybean nodules, nodule organogenesis completed in the initial stage when nodule diameter is about 1 mm and further nodule growth is mostly depending on the cell expansion rather than cell proliferation.

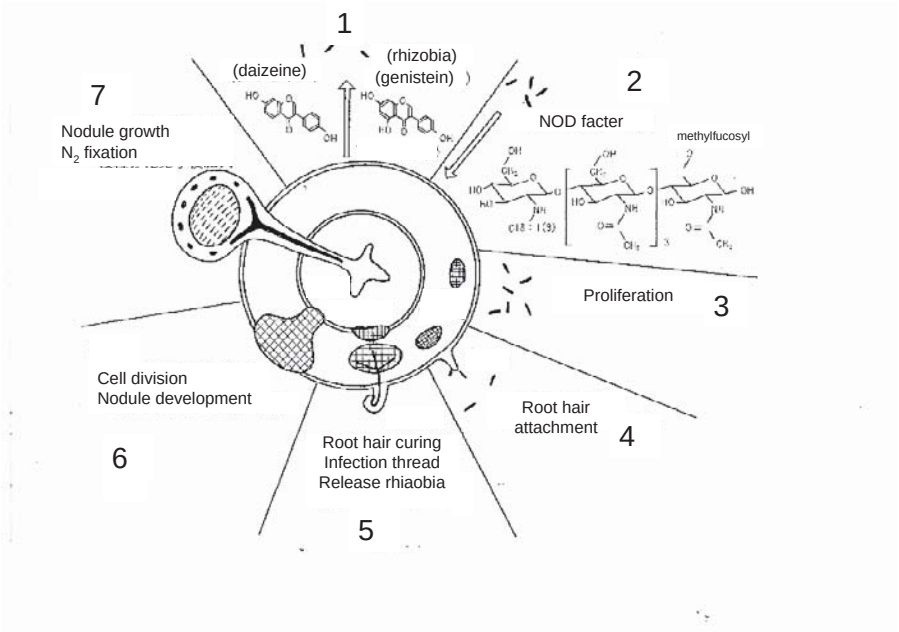


Figure 7. A model of infection and nodulation processes between soybean and rhizobia

Recently gene expression analysis can be applied for model legume *Lotus japonicus* by cDNA array analysis (Kouchi et al. 2004). Using the cDNA array of 18,144 non-retundant expressed sequence tags (ESTs) isolated from *L. japonicus*, and the expression of 1,076 genes was significantly accelerated during the successive stages after infection of compatible rhizobia, *Mesorhizobium loti*. These include 32 nodulin and nodulin-homolog genes as well as a number of genes involved in the catabolism of photosynthates and assimilation of fixed nitrogen. The gene expression profile in early stages of rhizobium-legume interaction was considerably different from that in subsequent nodule development. A number of genes involved in the defense responses to pathogens and other stresses were induced abundantly in the infection process, but their expressions was suppressed during subsequent nodule formation. The genome sequencing information and genome resources in model legumes, *L. japonicus* and *Medicago truncathula* have been available for many aspects of legume studies (Sato et al. 2007).

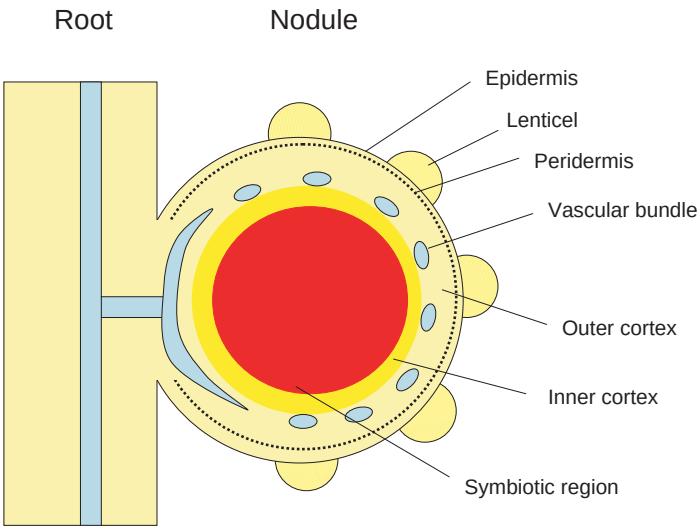


Figure 8. A model of structure of soybean root nodule

3. NODULE MORPHOLOGY OF SOYBEAN

Soybean nodule is classified to determinate type nodule, which has a spherical form, and nodule growth is mainly due to cell expansion after initial cell proliferation and development (Figures 8-10). The soybean nodule has the symbiotic region (or infected region in synonym) in the center, which consists the mosaic of large infected cells and small uninfected cells. The infected cells are filled with bacteroids (the symbiotic forms of rhizobia) and they are easily recognized by the red color with nodule specific protein, leghemoglobin (Lb) discovered by Kubo (1939). Lb is a most abundant protein in nodules (about 20% of total protein) and it can bind with O_2 to form LbO_2 to decrease free O_2 concentration in infected cells. The nitrogenase, an enzyme to fix N_2 in bacteroid, is very susceptible to free O_2 and irreversibly destroyed by O_2, therefore, free O_2 concentration should be kept very low in symbiotic region of nodules. On the other hand nitrogen fixation and assimilation processes require a large amount of energy and reductant produced by O_2 respiration, therefore, nodule respiration is about four times higher than that of roots based on dry weight. To support active

respiration, abundant supply of O_2 is essential. Lb in legume nodules solves the dilemma to keep free O_2 concentration low and sufficient supply of O_2 for bacteroid respiration.

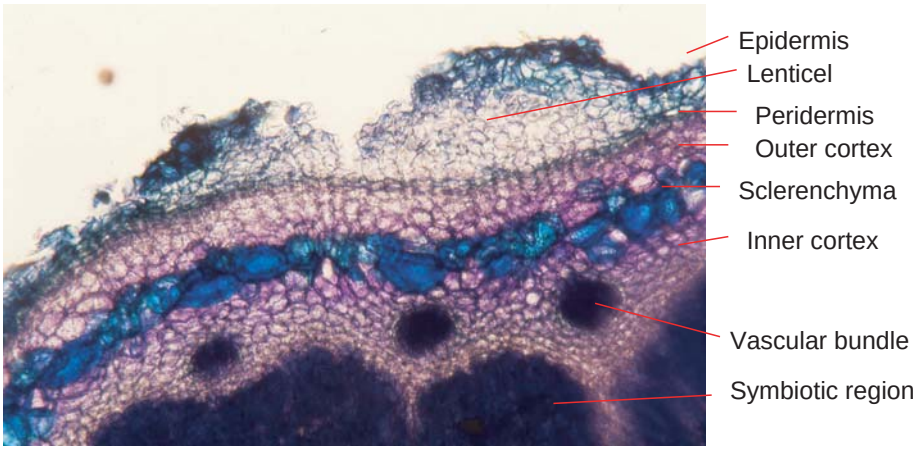


Figure 9. A model of structure of soybean root nodule stained with CBB From Mizukoshi et al. 1995

Symbiotic region is surrounded by nodule cortex in which the network of vascular bundles surrounding the symbiotic region to supply photoassimilate to bacteroids and to receive N fixation products from them (Figures 8, 9, 10). The nutrient sharing between symbionts was reviewed recently (White et al. 2007). Nodule cortex consists of inner cortex with small cells and outer cortex with large loosely packed cells. The sclerenchyma cells which have thick cell wall were located in the outer cortex (Figure 9). O_2 concentration decreases sharply through the inner cortex, and the O_2 permeability is flexibly controlled by this layer. It is hypothesized that a reversible exchange of intercellular water by the inner cortical cells plays a role in the regulation of nodule conductance to O_2 diffusion (Serraj et al. 1998). There are lenticels outside of nodules and one layer of epidermis. Under the epidermis, there is a peridermis, a tightly packed one layer of cells, which may restrict free diffusion of solutes between inside the nodule and medium solution (Mizukoshi et al. 1995).

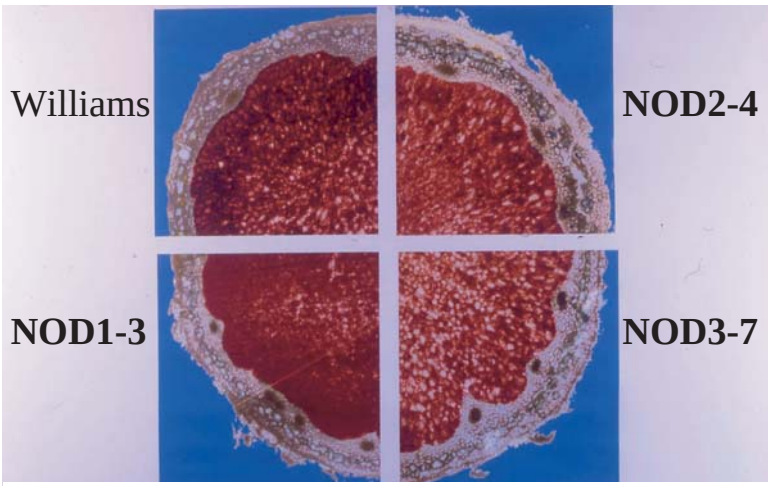


Figure 10. Nodule structure of wild type soybean cv. Williams and its hypernodulation mutant lines, NOD1-3, NOD2-4 and NOD3-7 From Nishiwaki et al. 1997

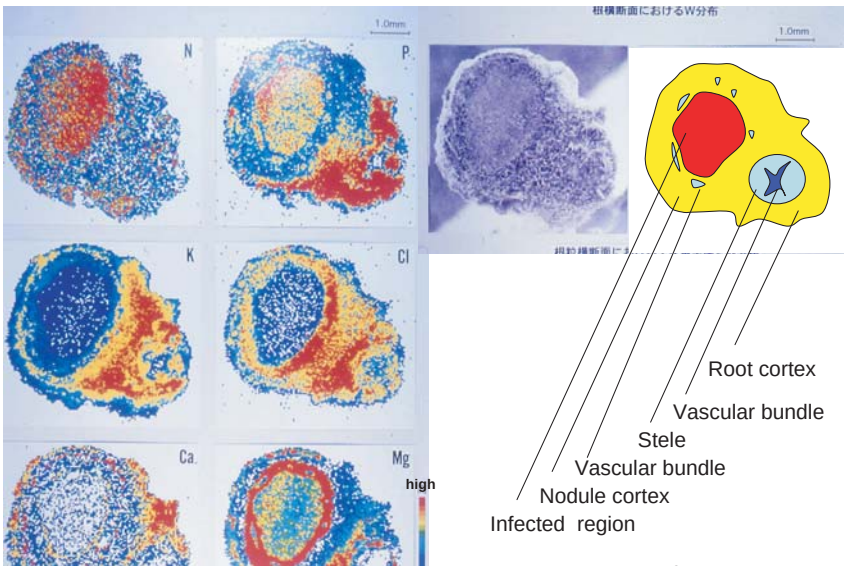


Figure 11. Distribution of N, P, K, Cl, Ca and Mg in the noduled root of structure of soybean. H: high concentration, L: low concentration From Mizukoshi et al. 1995

Soybean nodule is highly organized complex organ as shown by the distribution of minerals examined by EPMA (Electron Probe X-ray Microanalysis). Figure 11 shows the distribution of minerals in nodulated roots. The concentrations of N and P were higher but those of K and Cl were lower in the symbiotic region compared with nodule cortex. Ca was locally distributed in the surface layer, sclerenchyma cells and inner cortex, but the content was low in the symbiotic region. Mg specifically accumulated in the inner and outer cortex inside sclerenchyma cells but not outside them (Mizukoshi et al. 1995).

Chapter 2

THE FATE OF NITROGEN FIXED IN SOYBEEAN NODULES

1. INITIAL PROCESSES OF THE ASSIMILATION OF FIXED N IN SOYBEAN NODULES

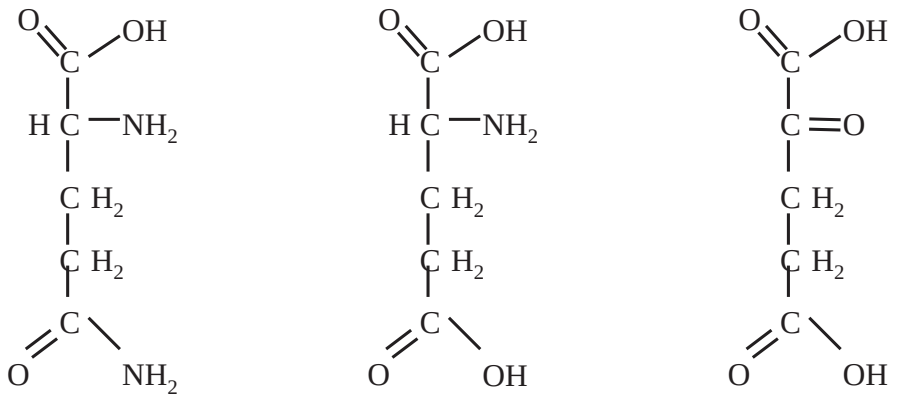
Ammonia is known to be the initial product of nitrogenase. Bergersen (1965) observed that after the exposure of $^{15}N_2$ to soybean nodules for 1 min, more than 90% of the fixed N in soluble fraction was detected as ammonia. The *Km* value (Michaelis cnstant) of N_2 for nitrogen fixation by purified nitrogenase was 8-16%, and that in the detached nodule in the air is 5% in the air and that in solution was 0.029 mole m^{-3} (Bergersen 1999). Until late 1970th, no direct evidence was obtained how the fixed N in bacteroids is transported to the host plant cytosol, and how it is metabolized to translocation forms of N to the shoots.

Figure 12 shows the structures of glutamine (Gln), glutamate (Glu) and 2-oxo-glutarate (2-OG). Until 1970, the fixed ammonia had been considered to be initially combined with 2-OG producing Glu catalyzed by glutamate dehydrogenenase (GDH) in plants and microbes. Not only ammonia produced by nitrogen fixation, the ammonia absorbed in the roots and reduced from nitrate was also believed to be assimilated by GDH enzyme.

GDH catalyzes the reaction as follows (Layzell 1990):

$$NH_4^+ + \text{2-oxoglutarate} + 2\ e^- \rightarrow \text{glutamate}$$

In roots or leaves, the enzyme is located in mitochondria or chloroplast, and the electron donor may be either NADH or NADPH.



glutamine **glutamate** **2-oxoglutarate**

Figure 12. Structure of glutamine, glutamate, and 2-oxoglutarate.

After discovering a new enzyme glutamate synthase (GOGAT) in *Aerobacter aerogenes* (Tempest et al. 1970), it is confirmed that ammonia can be assimilated via alternative of GDH, via glutamine synthetase (GS) and GOGAT pathway. Wolk et al (1976) demonstrated that the fixed ammonia is assimilated through GS/GOGAT pathway in nitrogen fixing blue green algae, *Anabaena cylindrica* by using ^{13}N as a tracer.

GS catalyzes the following reaction:

$$NH_4^+ + \text{glutamate} + ATP \rightarrow \text{glutamine} + ADP + Pi$$

This reaction requires divalent cation, such as Mg^{2+}, Mn^{2+} or Co^{2+} as a cofactor. There are a number of isoforms of GS, GS_1 (cytosolic) and GS_2 (chloroplast) forms of leaves, GS_r root cytosolic form, and GS_n nodule cytosolic form.

GOGAT catalyzes the following reaction:

$$\text{glutamine} + \text{2-oxoglutarate} + 2\ e^- \rightarrow 2\ \text{glutamate}$$

The electron donor is ferredoxin (Fd) or NADH in higher plants. In leaves most activity is Fd-dependent enzyme, and NADH-GOGAT locates in the plastid of non-photosynthetic tissues.

The net reaction of GS/GOGAT pathway is as follows:

$$NH_4^+ + \text{2-oxoglutarate} + 2\ e^- + ATP \rightarrow \text{glutamate} + ADP + Pi$$

Compared with GDH reaction, GS/GOGAT pathway requires one extra ATP as a substrate, and it means GS/GOGAT needs more energy than GDH. Although the higher cost of the ammonia assimilation by GS/GOGAT than GDH, the GS with lower *Km* for ammonia has an advantage to assimilate ammona at low concentration in cells before reaching toxic level.

Figure 13. Apparatus for $^{15}N_2$ feeding experiment with intact soybean plants.

To elucidate the initial ammonia assimilation pathway in soybean nodules, the intact nodules attached to the upper part of the roots were exposed to $^{15}N_2$ gas for 21 min (Figure 13) (Ohyama and Kumazawa 1978). $^{15}N_2$ gas was prepared from ^{15}N labeled ammonium sulfate then mixed with He and O_2 (^{15}

N_2:He:O_2=1:7:2) (Ohyama and Kumazawa 1981c). The 21 min of $^{15}N_2$ exposure was followed by non-labeled conditions for 29 min (chase period), and the nodules were harvested at 2, 4, 6,10, 15, 20, 25, 30, 35, 40, 50 min after starting $^{15}N_2$ exposure. The fresh nodules were rapidly homogenized with 80% ethanol, and ammonia, amino acids and ureides (allantoin and allatoic acid) were extracted. Figure 14 shows the structures of allantoin, allantoic acid, nitrate and asparagine. The incorporation of ^{15}N into various nitrogen compounds was determined by the optical emission spectrometry (Ohyama and Kumazawa 1979b, Ohyama 1982, Ohyama et al. 2004, FNCA 2006).

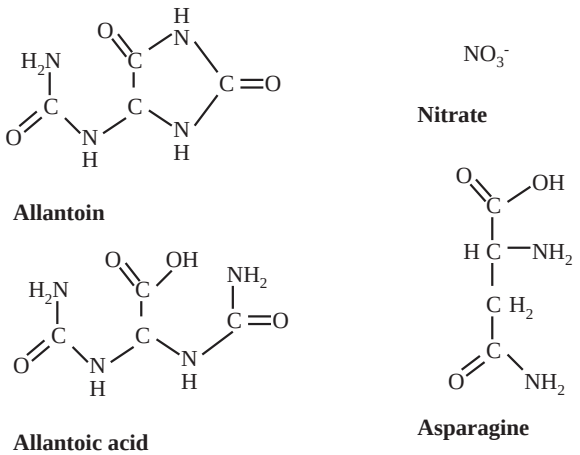


Figure 14. Structure of allantoin, allantoic acid, nitrate and asparagines.

Figure 15 shows the time course of ^{15}N incorporation into N compounds until 10 min, and Figure16 shows the time course including 21 min period of $^{15}N_2$ feeding and 29 min of chase period. In Figure 15, the ^{15}N abundance of ammonia increased rapidly after a few min of $^{15}N_2$ exposure, and soon reached the maximum value at about 0.5 % showing a hyperbolic curve. This suggests that there are two or more compartments of ammonia in nodules, and one of which may be the ammonia pool directly derived from nitrogen fixation. The size is relatively small, less than 1% of total ammonia in nodules, but the turnover rate is very rapid in a few min. Among amino acids, glutamine gave the highest ^{15}N abundance until 10 min of ^{15}N supply (Figure 15). When the ^{15}N abundance of amido-N and amino-N of glutamine was separately measured, the ^{15}N was rapidly incorporated into amido-N, then amino-N after a few min lag-time. Following glutamine, glutamic acid and alanine increased ^{15}N abundance. After changing gas

phase from $^{15}N_2$ to non-labeled conditions (Figure 16), ammonia and glutamine showed the immediate decrease of ^{15}N abundance, this indicates the characteristics of primary assimilatory products. On the other hand, the ^{15}N abundance of glutamate alanine continue to increase for a few min after changing to non-labeled conditions. ^{15}N was relatively rapidly incorporated into ureides (the sum of allantoin and allantoic acid) in 10 min of $^{15}N_2$ exposure (Figure 15), although the ^{15}N abundance did not decrease during chase period (Figure16). This data is the first experimental evidence that ureides are synthesized actively in soybean nodules from fixed nitrogen. The incorporation of ^{15}N was faster in allantoin than allantoic acid, suggesting that allantoic acid is formed from allnatoin in nodules, and not vise verse (Ohyama and Kumazawa, 1978). The ^{15}N was slowly incorporated into asparagine, although the time lag was longer than ureides.

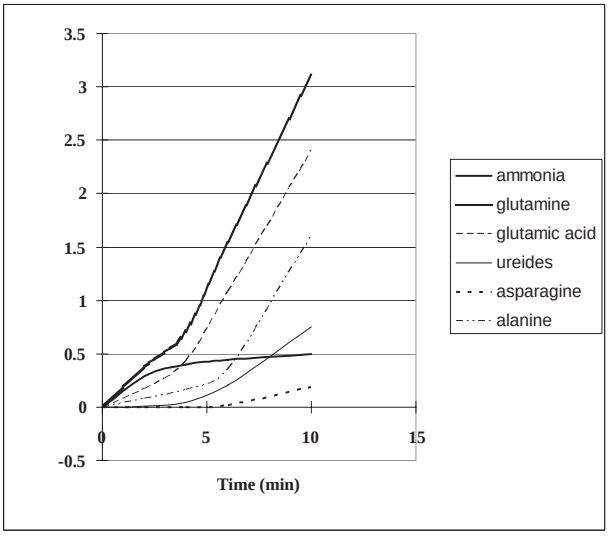


Figure 15. Time course of ^{15}N labeling in soybean nodules after $^{15}N_2$ exposure for 10 miniutes. From Ohyama and Kumazawa 1978

From the result obtained by the $^{15}N_2$ pulse chase experiment, the ammonia fixed by nitrogenase in bacteroids is located in a small compartment compared with whole nodule ammonia pool. The rapid incorporation of ^{15}N into glutamine especially the amido-N, followed by glutamate, and amino-N of glutamine in this sequence was in accordance with the initial assimilatory pathway be GS/GOGAT pathway rather than GDH. This was supported by the evidence that the rapid

decline of ^{15}N in glutamine but not glutamate immediately after changing to the chase period.

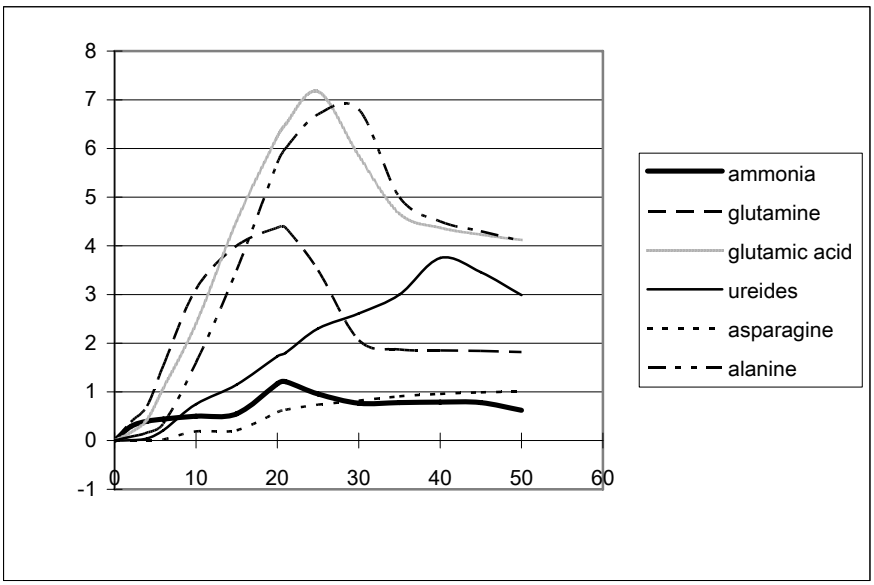


Figure 16. Time course of ^{15}N labeling in soybean nodules after 21 minutes of $^{15}N_2$ exposure and hase period for 29 minuites. From Ohyama and Kumazawa 1978

The assimilatory pathway of ammonia produced by N_2 fixation was confirmed by using specific metabolic inhibitors, methionine sulfoximine (MSX) for GS and azaserine (AS) for GOGAT (Ohyama and Kumazawa 1980a). The MSX or AS solution was injected into intact nodules, and $^{15}N_2$ was exposed to the nodules for one hr. The MSX treatment increased the ^{15}N abundance of ammonia more than 6 times, however, it depressed the ^{15}N abundance of amido-N of glutamine and amino acids. In addition, the AS treatment increased the ^{15}N content of ammonia and amido-N of glutamie, but it decreased the amino-N of glutamine and other amino acids. Experiments with nodule slices pretreated with MSX or AS solution, then they were fed with ^{15}N labeled ammonia or amido-^{15}N of glutamine showed the same trends (Ohyama and Kumazawa 1980a). These results strongly support that the ammonia produced by nitrogen fixation in soybean nodules is mainly assimilated by GS/GOGAT pathway and not by GDH.

2. N TRANSFER FROM BACTEROID TO CYTOSOL

In another experiment, fresh nodules are macerated in 0.2M sorbitol solution at 4 ^{o}C, and separated into bacteroid and cytosol fractions by centrifugation at 6000g for 15 min after filtration through nylon net. Each fraction was extracted with 80% ethanol, and the concentration of N compounds was determined. Figure 17 shows the comparison of the N concentration of major compounds in bacteroid and cytosol fractions. Two fractions gave quite different patterns of composition. In cytosol, asparagine was most dominant compound, followed by ureides and other amino acids. In bacteroid, ammonia concentration was relatively high, and the concentration of ureides and asparagine was negligible. Among amino acids, glutamate, alanine, 4-aminobutylate (GABA) were major compounds in bacteroid fraction (Ohyama and Kumazawa 1978).

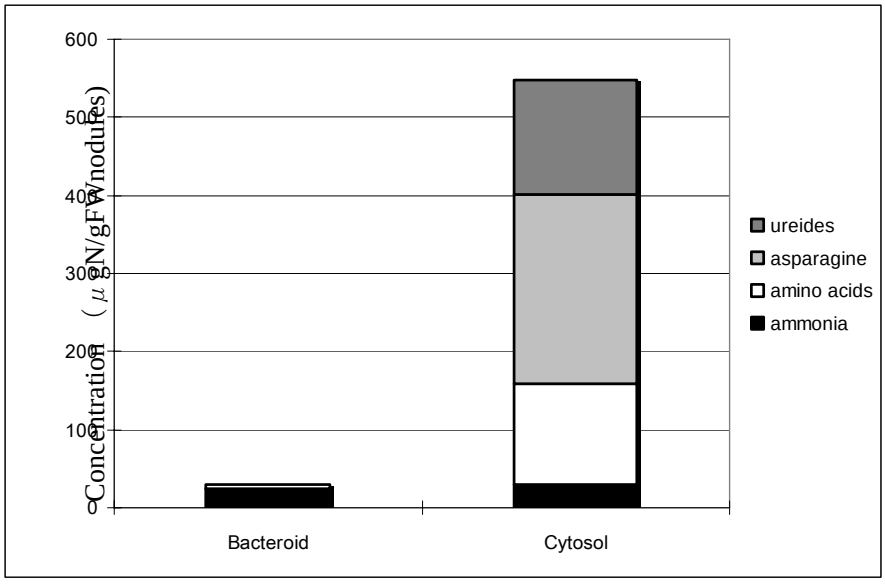


Figure 17. Major N constituents in bacteroid and cytosol fractions of soybean nodules. From Ohyama and Kumazawa 1978

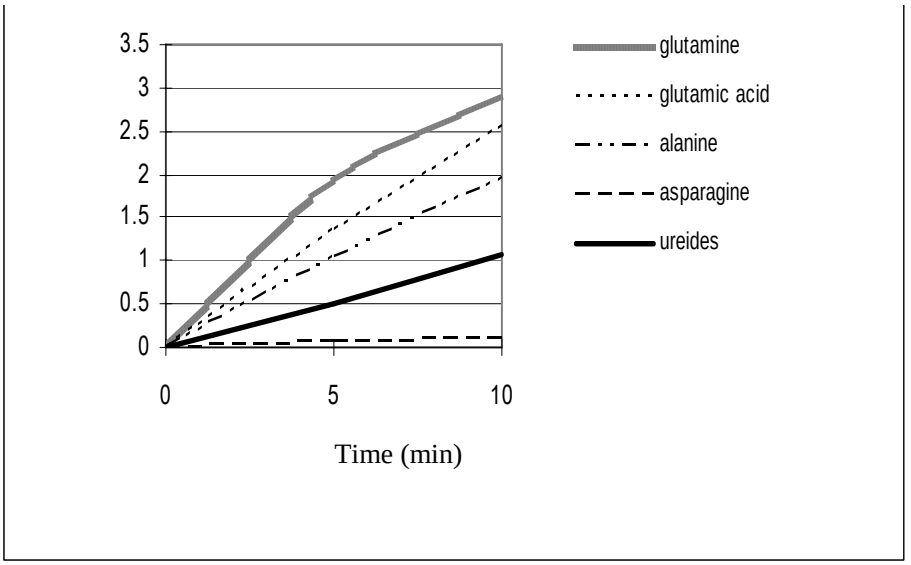


Figure 18. Time course of ^{15}N labeling in cytosol fraction of soybean nodule after $^{15}N_2$ exposure for 10 minuites. From Ohyama and Kumazawa 1980b

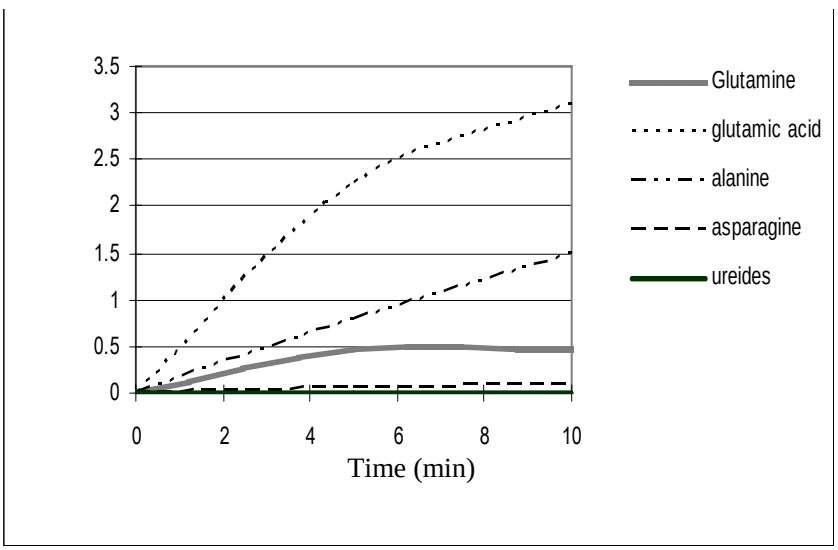


Figure 19. Time course of ^{15}N labeling in bacteroid fraction of soybean nodule after $^{15}N_2$ exposure for 10 minuites. From Ohyama and Kumazawa 1980b

^{15}N assimilation was investigated in cytosol (Figure 18) and bacteroid (Figure 19) fractions of soybean nodules (Ohyama and Kumazawa 1980b). $^{15}N_2$ was exposed to intact soybean nodules for 5 and 10 min, and bacteroid and cytosol fractions were immediately separated by centrifugation. Although $^{15}N_2$ feeding period was very short, most of the ^{15}N was located in cytosol fraction, and only 3-4% of soluble ^{15}N remained in bacteroid fraction. As shown in Figure 18, in cytosol the ^{15}N abundance of glutamine was the highest and followed by glutamate, alanine and ureides in this sequence. In bacteroid fraction (Figure 19), glutamate showed the highest ^{15}N abundance followed by alanine and aspartate, but ^{15}N incorporation was very slow into glutamine. When 1 mM $^{15}NH_4^+$ was supplied to the bacteroid suspension for 15 min, ^{15}N was rapidly incorporated into glutamate and alanine, and glutamine was not labeled during this period (Ohyama and Kumazawa 1980b).

3. PRIMARY EXPORT FORM OF FIXED NITROGEN FROM BACTEROID IS AMMONIA NOT ALANINE

From these results, it was suggested that most of the fixed N is immediately exported from bacteroid to cytosol and assimilated via GS/GOGAT pathway in cytosol, then metabolized into various amino acids via transamination from glutamate. Ureides, allantoin and allantoic acid are synthesized from amino acids and amides (Figure 20). On the other hand, a small part of fixed N is metabolized in bacteroids, and it is not by GS/GOGAT pathway but probably by GDH or alanine dehydrogenase (ADH). Waters et al. (1998, 2000) proposed an alternative N export from soybean bacteroid. They indicated that alanine not ammonia is the main export compound from bacteroid to cytosol, based on the data that the isolated bacteroid excrete ^{15}N labeled alanine when the suspension was exposed to $^{15}N_2$ gas. However, Li et al. (2001) support the ammonia is the main export compounds from soybean bacteroid, by careful preparation of bacteroid suspension under optimum $^{15}N_2$ feeding conditions. Our previous results clearly showed that bacteroid can synthesis alanine through GDH or ADH, but ammnonia is a major export compound from bacteroid to cytosol.

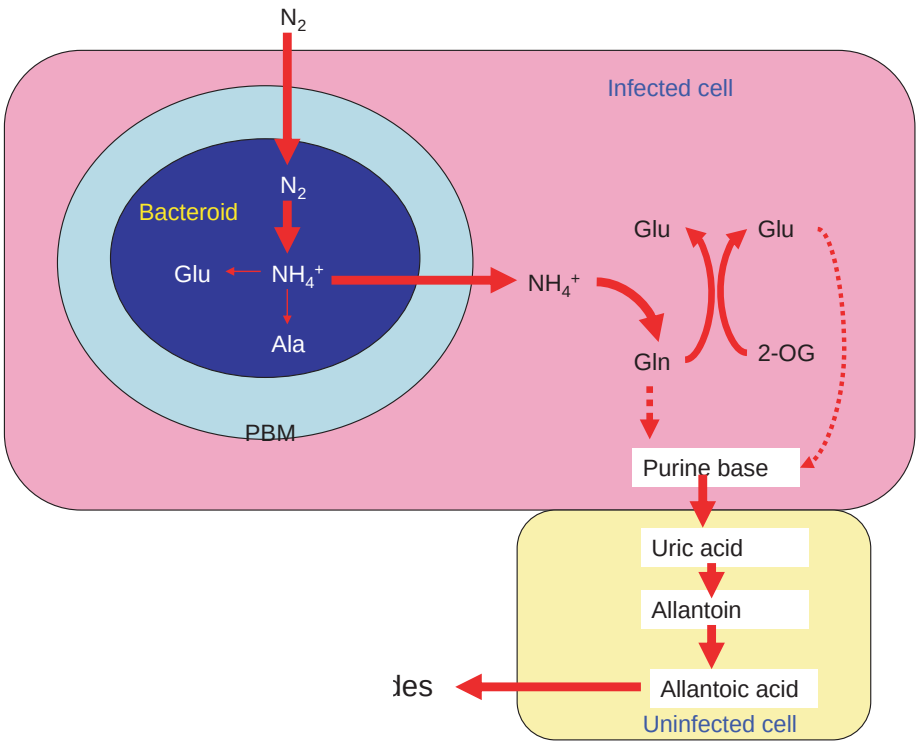


Figure 20. Nitrogen metabolism in soybean nodules

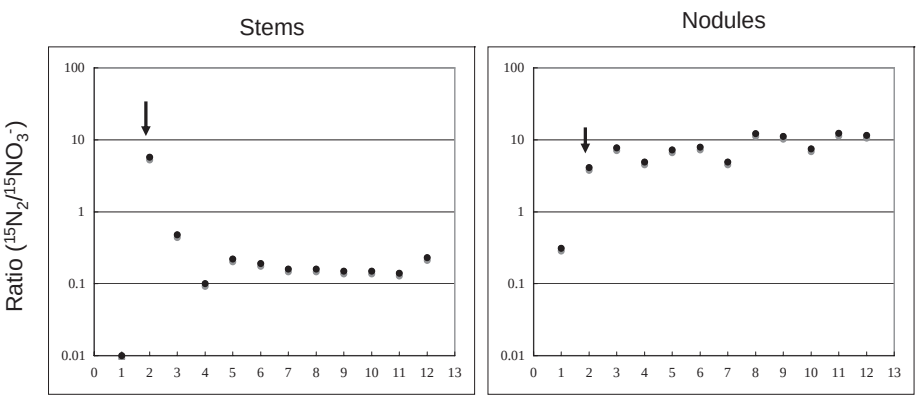


Figure 21. Comparison of the ratios of 15N abundance from 15N2/15NO3- in stems and nodules. 1: nitrate, 2: ureides (arrow), 3: ammonia, 4: glutamine, 5: glutamate, 6: asparagine, 7: aspartate, 8: alanine, 9: 4-aminobutylate, 10: serine, 11: isoleucine, leucine, 12: proline From Ohyama and Kumazawa 1979a

4. UREIDE SYNTHESIS IN SOYBEAN

Some leguminous plants including soybean transport the fixed N mainly in the form of ureides (allantoin and allantoate). All the species in Phaseoleae (soybean, common bean, cowpea etc.) and some species in Robinieae, Indigoforeae and Desmodieae transport ureides (Atkins 1991). Ishizuka et al. discovered that nodulated soybean plants contain a large amount of ureides in stems, while non-nodulating isoline contain much less amount of ureides (Kushizaki et al. 1964). They hypothesized that ureides may be synthesized in the root part where the metabolism might be disturbed by nodulation. On the other hand, Matsumoto et al. (1975) suggested the site of ureide formation may be in nodules and not in roots of soybean. Reviews on ureide biosynthesis in legume nodules were published (Scubert 1986, Tajima et al. 2004).

We compared the labeling patterns of ureides and amino acids from $^{15}N_2$ and $^{15}NO_3^-$ (Ohyama and Kumazawa 1979ab). One group of soybean plants was exposed to $^{15}N_2$ and non-labeled NO_3^-, and another group was fed with $^{15}NO_3^-$ solution with non-labeled N_2. After 8 hrs of treatments soybean plants were harvested, and the ^{15}N abundance of ureides and ammonia, nitrate, and amino acids were determined. The ratios of ^{15}N abundance from $^{15}N_2$ and $^{15}NO_3^-$ in stems and nodules were calculated (Figure 21). The ratio of ureides (about 6) in stems showed 10-50 fold higher than the ratios of other compounds (0.1-0.48), indicating that most of ureides derived from fixed N rather than absorbed N. When the same ratios were calculated in nodules, the ratios are mostly the same including ureides except for nitrate. This suggested that ureides are mainly synthesized in nodules either from fixed N_2 or from absorbed NO_3^- but only small amount of ureides are synthesized in the root part.

The experiment above was compared the metabolism of fixed N and absorbed NO_3^- in relatively short term, however, the same trends were observed by the long term experiment in which soybean plants were cultivated with continuous supply of $^{15}NO_3^-$. Soybean plants were cultivated with hydroponics, and $^{15}NO_3^-$ was supplied in the culture solution (Ohyama et al. 1981a). Soybean plants were harvested at the initial flowering stage (R1) and initial pod filling stage (R3), and the percentage of N derived from $^{15}NO_3^-$ was determined by ^{15}N analysis (R shows the reproductive stage proposed by Fher et al. 1971). The percentage of nitrogen originating from $^{15}NO_3^-$ in the stems, petioles, and leaf blades were almost the same as in the nodules in both stages (about 10% at R1 and about 8% at R3). However, the percentage in roots was higher about 20% at R1 and 14% at R3 stage. These results indicated that most of ureides in shoots derived from nodules,

and small portion of ureides is synthesized in the roots but it contributes very little to nitrogen transport to the shoots.

The effects of various nitrogen fertilizers on the concentrations of ureides and amino acids in the shoot were investigated in relation to nodule formation (Ohyama et al. 1981a). The concentration of amino acids was unaffected by nodulation. A low level of ureides was detected in all the plants including non-nodulatated ones, and after a certain critical stage of nodule growth, the ureide content increased in proportion to the increase in nodule fresh weight.

When the nodule slices were fed with ^{15}N labeled precursors of purine biosynthesis, glutamine and glycine, for 30 min, ^{15}N incorporation into ureides were observed (Ohyama and Kumazawa 1981b). In addition, from ^{14}C labeled hypoxanthine feeding the radioactivity was detected in the Rf of xanthine, uric acid, allantoin and allantoic acid separated by paper chromatography (Ohyama and Kumazawa 1981b). Furthermore, a low O_2 condition depressed the ureide synthesis in intact soybean nodules. These results are in accordance with the ureide synthesis pathway through oxidative degradation of purine base.

The reason why nodules synthesize ureides although roots synthesize asparagine is not fully understood yet. The ureides contain 4N with 4C in a molecule and which is more efficient to transport form of N compared with asparagine with 2N and 4C in a molecule. It is postulated that ureide synthesis in nodule may be evolved to adapt to economical use of C because nodule needs a lot of carbon source for N_2 fixation and assimilation. The trigar to accelerate ureide synthesis has not know yet, but the high N or low C concentrations, low pO_2 may be involved. Chen et al. (1999) investigated the profile of ureide accumulation in various tissue of alfalfa which is amide type legume. In alfalfa plants the ureide concentration was higher in the lateral roots and nodules than in other tissues. In the main root the concentration of ureides increased gradually towards the root tip.

Figure 22 shows the metabolic pathways and transport of N derived from N_2 fixation and NO_3^- absorption in soybean plants. The N fixed by the bacteroid is rapidly exported to the plant cytosol as in the form of ammonium, then the NH_4^+ is assimilated into glutamine by GS in the cytosol of infected cells. Then glutamine is converted to 2 glutamate in the plastid via GOGAT. Then xanthine and uric acid is formed by purine degradation, and uric acid is transported to the neibering uninfected cells, then it is further degraded to allantoin and allantoic acid. On the other hand, some part of the NO_3^- absorbed in the roots are reduced in the roots to NO_2^- by nitrate reductase, then the NO_2^- is further reduced to NH_4^+ by plastidic nitrite reductase. Then the NH_4^+ is assimilated by GS/GOGAT pathway in the roots, and metabolized to asparagine then transported to shoot via xylem.

Some part of NO_3^- is directly transported through xylem to the shoots. Ohtake et al. (1995) reported the seasonal changes in amino acid composition in xylem sap of soybean and they confirmed that asparagine was the principal amino acids in xylem sap at any stages. Masuda et al. (2003) compared the concentration of amino acids and ureides and the correlation was observed in non-nodulated soybean but it was not observed in nodulated soybean cultivated in the field.

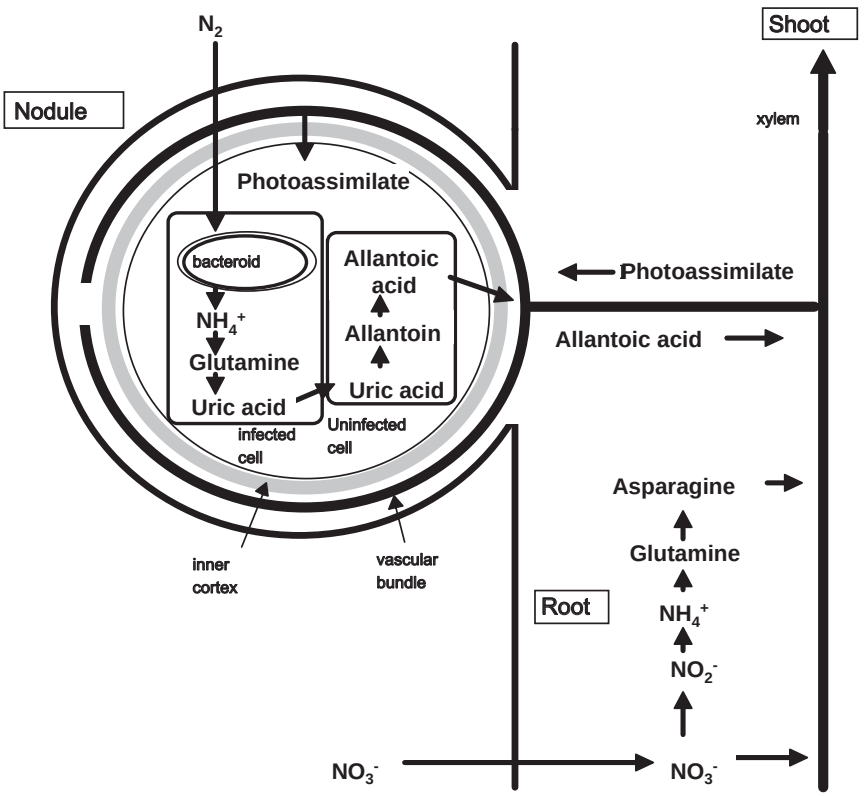


Figure 22. Nitrogen metabolism and transport in nodules and roots of soybean plants.

5. NITROGEN METABOLISM IN SOYBEAN LEAVES

Plant leaves are the important organ for nitrogen metabolism, as well as photosynthesis. The N absorbed from roots or fixed in root nodules is transported via xylem in stems and petioles, and the N is translocated to the sink organ such as pods and seeds via phloem. The flow of N in leaves was investigated by petiole

girdling and $^{15}N_2$ or $^{15}NO_3^-$ feeding experiment (Ohyama and Kawai 1983). The petioles of the upper four leaves were girdled with hot steam. One hr after girdling, $^{15}N_2$ or $^{15}NO_3^-$ were fed for 10 hr, and the plants were harvested 24 hr after $^{15}N_2$ or $^{15}NO_3^-$ treatment. Compared with control leaves without girdling, sugar concentration increased in the girdled leaves, such as fructose (x 3.5) glucose (x 3.2), and sucrose (x 1.8) due to blocking of phloem transport. Fellows et al. (1978) reported that sucrose is a major photoassimlate from leaves to pod via phloem by the pod leakage technique in soybean. Concerning to N compounds, the accumulation of amino acids (x 2.5) especially asparagine (x 8.8) was observed, indicating that these compounds are the major transport forms from leaves to sink organ via phloem.

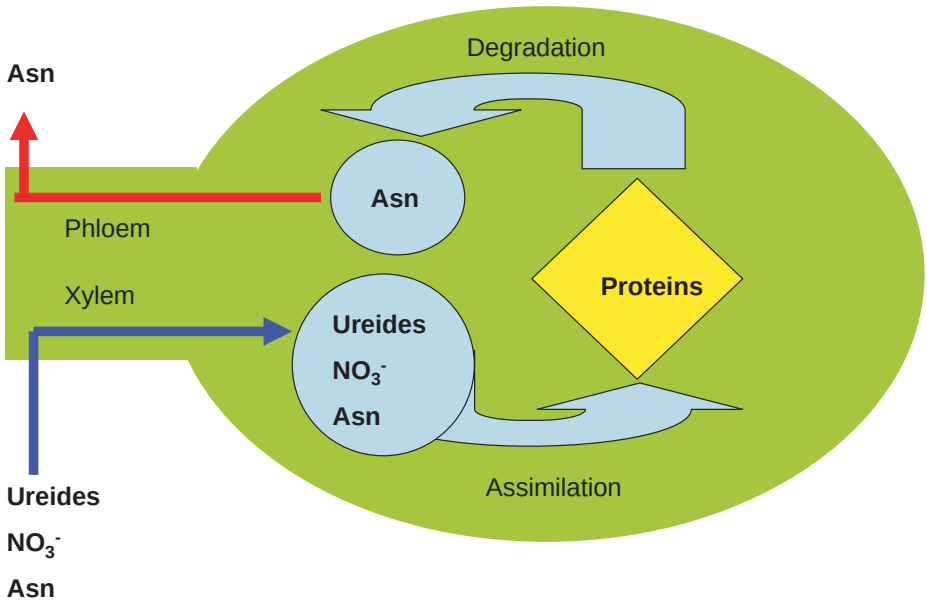


Figure 23. A model of N flow in soybean leaves.

However, nitrate and ureides remained in the same level as non-girdled leaves and they are not accumulated in girdled leaves, suggesting that nitrate and ureides are not transported from leaves to sink via phloem (Figure 23). There are tow different ureide degradation pathways in soybean leaves, either by allantoate amidinohydrolase or by allantoate amidohydrolase (Vadez and Sinclair, 2000).

6. COMPARISON OF NITROGEN ALLOCATION DERIVED FROM FIXED N_2 AND FROM ABSORBED NO_3^-

Comparative study on the nitrogen metabolism and transport was done at the pod filling stage (Ohyama 1983). Nodulated soybean plants were hydroponically cultivated with 0.7 mM nitrate, and $^{15}N_2$ or $^{15}NO_3^-$ treatment was conducted for 10 hr. Immediately after 10 hr of $^{15}N_2$ treatment, the 36% of fixed N remained in nodules, then the rest was located in roots (9%), stems (17%), leaves (18%), pods (10%), and seeds (10%). During chase period of five consecutive days under non-labeled conditions, ^{15}N content decreased in the nodules, roots and stems and the corresponding amount of ^{15}N was translocated to the seeds (36% at 5^{th} day). At the end of 10 hr of $^{15}NO_3^-$ treatment, 36% of absorbed ^{15}N was remained in the roots, and the rest was located in nodules (0.4%), stems (17%), leaves (36%), pods (5%), and seeds (5%), respectively. During chase period of 5 consecutive days under non-labeled conditions, ^{15}N content decreased in the roots, stems and leaves, and the corresponding amount of ^{15}N was translocated to the seeds (44% at 5^{th} day).

Based on the results obtained, it was concluded that the fates of N derived from nitrogen fixation and N derived from nitrate absorption was different, although both N sources can be utilized either for vegetative or reproductive growth. Some portion of N derived from N_2 fixation is rapidly transported to the pods and seeds, while another portion was derived from N once assimilated in the nodules and other vegetative parts. On the other hand, most of N originated from NO_3^- was immediately assimilated into the proteins of roots and leaves, then the N was resolublilized by protein degradation and redistributed to the pods and seeds.

Sato et al. (1999) analyzed nitrate absorption and distribution in nodulated (T202) and non-nodulated (T201) soybean plants with $^{13}NO_3^-$ and $^{15}NO_3^-$. Real time observation of the accumulation pattern of ^{13}N was monitored by positoron emitting tracer imaging system (PETIS). The results showed that nitrate absorption and intial translocation pattern was not affected by nodulation.

We proposed a model of N flow derived from N_2 and NO_3^- in soybean plants as in Figure 24 (Ohyama 1984). The N derived from N_2 fixed by the root nodules is rapidly assimilated into ureides (allantoin and allantoate), and some ureides are directly transported to pods and used for seed development. Ureides are also used for leaf protein synthesis, but the contribution is relatively lower than N derived from NO_3^- absorbed from the roots. On the other hand, some part of NO_3^- absorbed from the roots is immediately reduced in the roots, and transported in the form of amino acids, especially asparagine. Another part of NO_3 is transported to

the leaf blades via transpiration, and assimilated into leaf protein. The remobilization of storage protein in leaves and roots may be a major source for seed N source in the case of NO_3^- nutrition. The similar trends were reported in two nodulated West African geocarpic legumes, Kersting's bean (*Macrotyloma geocarpum* L.) and Bambara groundnut (*Vigna subterranea* L.) (Dakora et al. 1992). The root xylem bleeding sap of both species showed ureides as predominant solute of nitrogen over 90%, when plants were relying solely on atmospheric N_2. With increasing the level of nitrate supply, the levels of ureide and glutamine decreased and those of asparagine and nitrate in xylem sap increased.

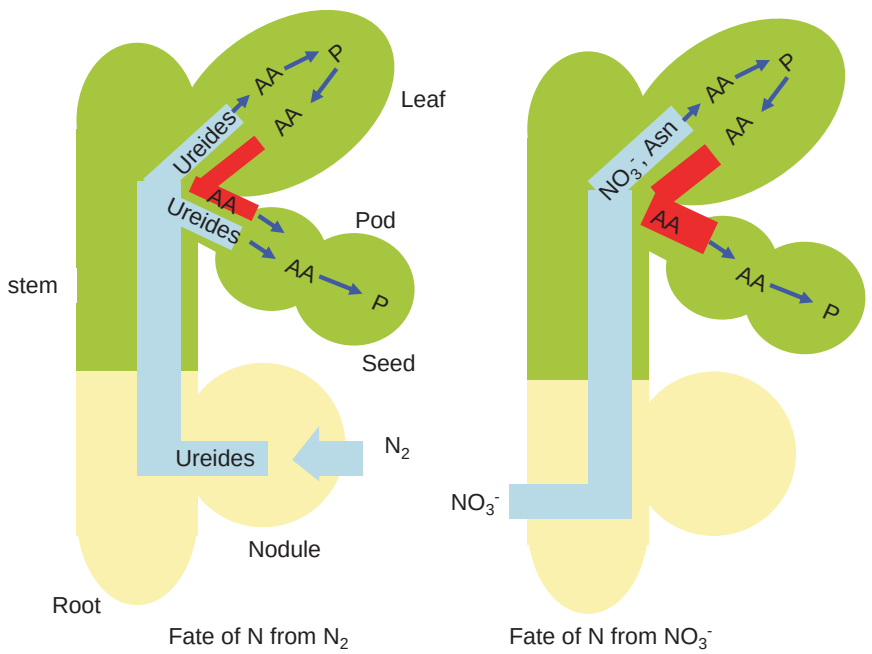


Figure 24. Models of transport of N from N_2 fixation and NO_3^- absorption in soybean plants. AA: amino acids, Asn: asparagine, P: protein

Chapter 3

CHARACTERISTICS OF NITRATE ABSORPTION AND TRANSPORT IN SOYBEAN PLANTS

1. ABSORPTION AND ASSIMILATION OF AMMONIUM AND NITRATE IN PLANT CELL

The outlines of absorption and metabolism of ammonium and nitrate in plant cells are shown in Fig. 25. Ammonium which is most reduced form of nitrogen and nitrate which is most oxidized form of nitrogen are two major inorganic nitrogen compounds in soil. The NH_4^+ ion is absorbed through the membrane bound protein, ammonium transporter. The NO_3^- ion is absorbed through the nitrate transporter with $2H^+$ co-transport. There are two types of nitrate transporter, a high affinity nitrate transporter system (HATS) and a low affinity nitrate transporter system (LATS) (Crawford and Glass 1998).

2. CHARACTERISTICS OF NITRATE ABSORPTION IN SOYBEAN ROOTS

The changes in NO_3^- concentration in solution were monitored by nutrient solution circulation system in which the UV absorbance was detected by UV detector (Figure 26). The relationship between nitrate absorption rate and nitrate

concentration was shown as Figure 27. The kinetics of this pattern indicates the presence of only one HATS which Km value is 19 μ mole in soybean roots. The value is comparable to that in potato cultivars from11.2-17.3 μ mole (Sharifi and Zebarth 2006).

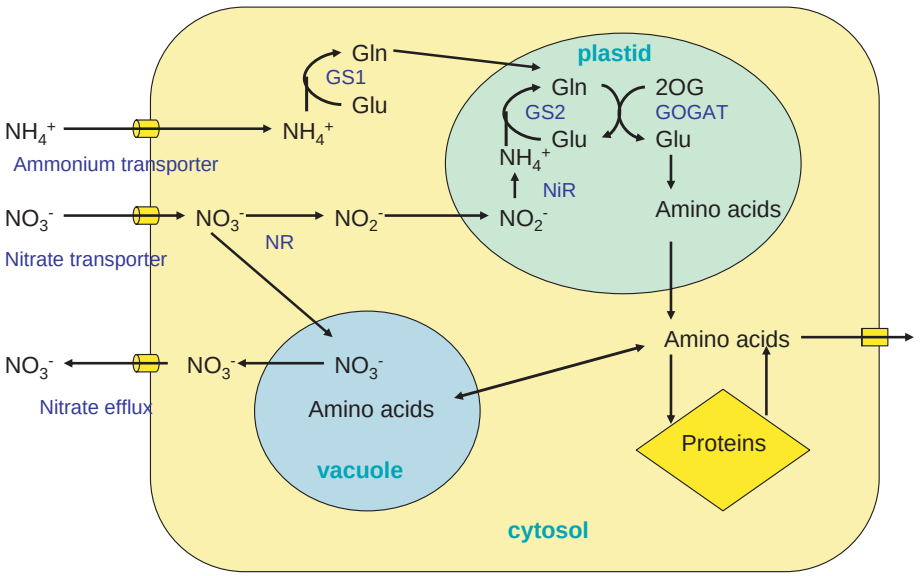


Figure 25. A model of absorption and metabolism of ammonium and nitrate in plant cell. Gln: glutamine, Glu: glutamate, GOGAT, glutamate synthase, GS1: glutamine synthetase (cytosol), GS2: glutamine synthetase (plastid), NR nitrate reductase, NiR: nitrite reductase,

Using the same system the characteristics of NO_3^- absorption was investigated. Figure 28 shows the effect of pH of the solution from 5 to 8 on NO_3^- absorption rate. The NO_3^- absorption rate was highest at pH 5, and decreased with increasing pH. At pH 8, NO_3^- absorption was stopped or some in roots was excreted to the solution. This result was in accordance with H^+ co-transport of NO_3^- absorption, because the higher the pH the lower the $[H^+]$ concentration (electrochemical potential). Figure 29 shows the NO_3^- absorption rate under medium temperature from 15 to 45 ^oC. At low temperature 15 ^oC, NO_3^- absorption rate was low only 20% of that at 25 ^oC. The NO_3^- absorpton rate increased at 35 ^oC, but it completely stopped at 45 ^oC. Concerning to temperature effect, this experiment was conducted just after changing the temperature from room temperature around 25 ^oC. After several hours of incubation at 45 ^oC, the NO_3^- absorption recovered by adaptation to high temperature.

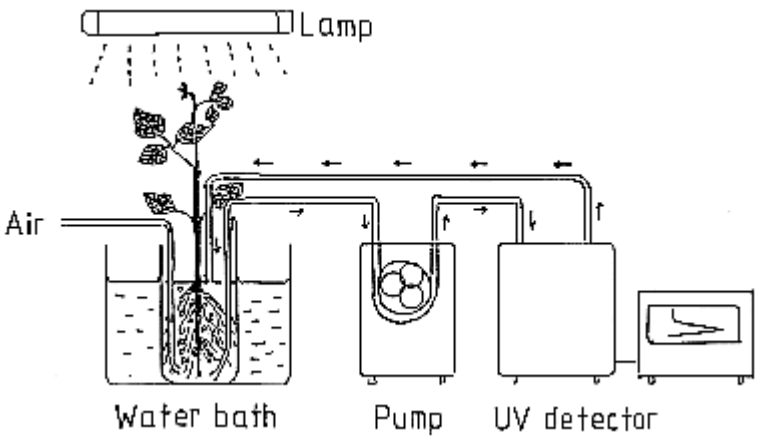


Figure 26. Circulation system for detecting NO_3^- concentration in culture solution. From Ohyama et al. 1989b.

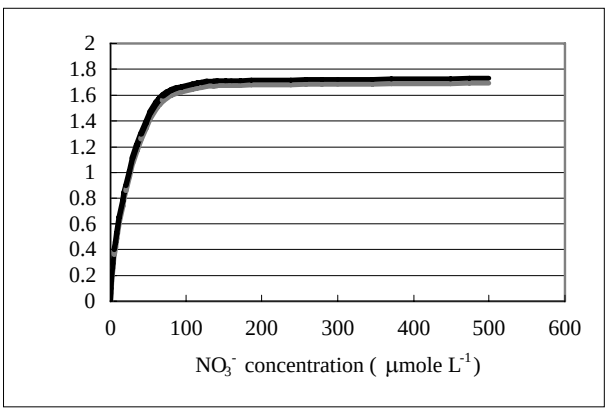


Figure 27. NO_3^- absorption rate vs NO_3^- concentration in culture solution

The diurnal rhythm in NO_3^- absorption by intact soybean plants was investigated by sampling the culture solution every 15 min and analyzed by ion chromatography (Ohyama et al. 1989b). When 10 mgN of nitrate was initially supplied in the solution, the soybean absorbed NO_3^- almost linearly irrespective of the time during the day and night period or NO_3^- concentration in the medium (Fig. 30). The NO_3^- absorption rate was different between day (1.10 mgN L^{-1} h^{-1}) and night period (0.77 mgN L^{-1} h^{-1}), and the level of the absorption rate in the night was about 60-75% of that in the daytime. The temporary interruption of NO_3^-

absorption was observed twice a day at dawn and dusk. The same trends were observed when the solution with 25 mgN L^{-1} of nitrate was supplied. The absorption rate during day time was 0.93 mgN L^{-1} h^{-1}, and that during night time was 0.68 mgN L^{-1} h^{-1}. It was suggested that this rhythmic pattern of NO_3^- absorption was not directly controlled by the shoots, because the rhythm continued under the extended dark period (Figure 31) or by cutting the shoots (Ohyama et al 1989b). When the roots were put in the water bath under constant temperature at 30 ^oC, the rhythm of NO_3^- absorption disappeared (Figure 32), suggesting that the nitrate absorption rate of soybean roots is controlled by monitoring the root temperature changes. Different results were reported by Delhon et al (1995a, 1995b). They reported diurnal regulation of NO_3^- uptake in soybean plants, and the NO_3^- absorption was monitored in the non-nodulated soybean plant during 14/10h light/dark period at a constant temperature of 26 ^oC. During the night NO_3^- uptake rate and nitrate reduction was decreased. The accumulation of NO_3^- and asparagine were observed in the roots in dark period.

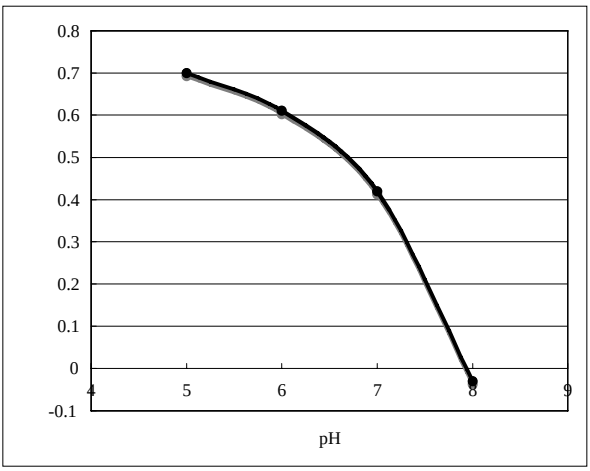


Figure 28. NO_3^- absorption rate under different pH conditions of culture solution.

When 0.5 mM KCN, which inhibit O_2 respiration, was added to the solution, NO_3^- absorption was completely stopped after a time-lag of about 1 hr (Ohyama et al. 1989b). The result suggests that NO_3^- absorption is active transport, which depends on the energy supply through O_2 respiration. The NO_3^- absorption was immediately stopped by the addition of 0.5mM CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) to culture solution, which chancel the electrochemical potential of H^+ across plasmamembrane (Finean et al. 1984). This confirms NO_3^-

transport across plasmamembrane is supported by H^+ motive force from appoplast to inside the cell through co-transport with H^+.

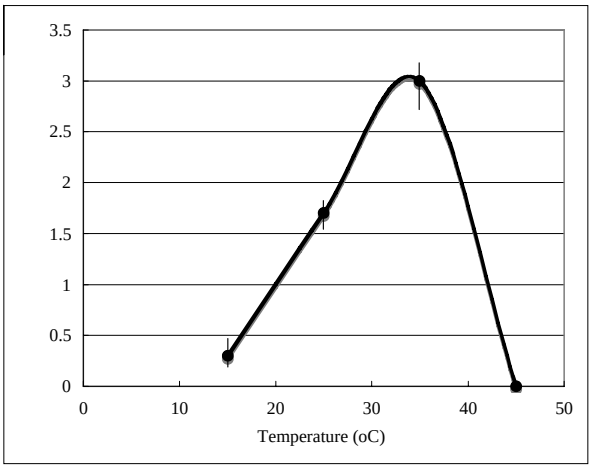


Figure 29. NO_3^- absorption rate under different temperature conditions of culture solution.

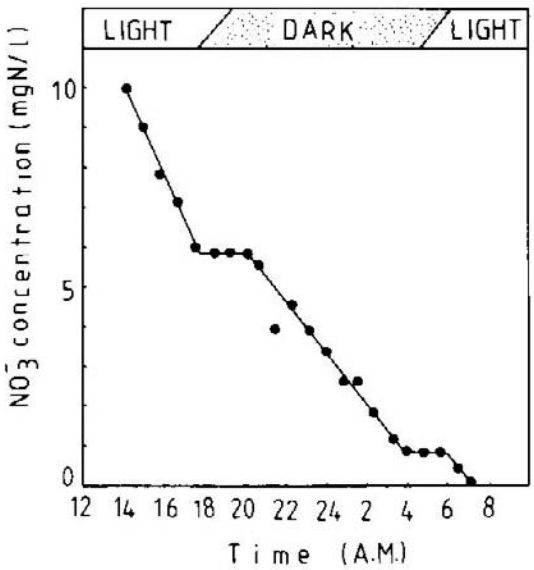


Figure 30. Diurnal changes in NO_3^- absorption rate by soybean root from culture solution. From Ohyama et al. 1989b

When 1mM ammonium was added to the solution, the NO_3^- absorption rate reduced to about 33% of the initial rate, after a lag-phase of 1 hr (Ohyama et al. 1989b). This result indicates that ammonium ion does not directly inhibit NO_3^- absorption, but the accumulation of assimilated N may cause the reduction of NO_3^- absorption rate as suggested by dwarf bean (Breteler and Siegerist 1984). On the other hand, when 1mM NO_2^- was added to the solution, the NO_3^- absorption was completely inhibited immediately.

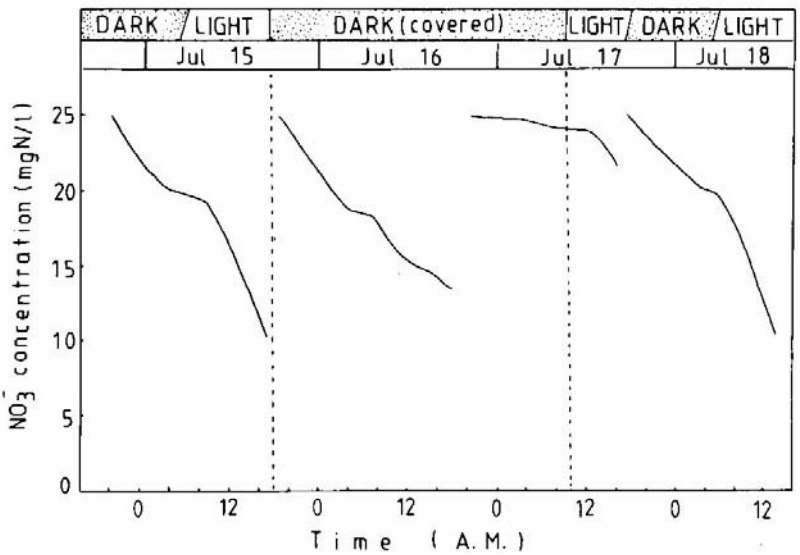


Figure 31. NO_3^- absorption rate by soybean root from culture solution under normal day/nignt cycle and continuous dark conditions. From Ohyama et al. 1989b

Some of the absorbed NO_3^- is known to excrete from cell to apoplast, which is called nitrate efflux. Efflux from soybean roots occurred only in the presence of NO_3^- in the medium (Ohyama et al. 1989b). The precise mechanism of nitrate efflux has not been understood yet, but this process may play a role in fine tuning of net NO_3^- absorption rate, which means influx rate minus efflux rate. The efflux of NO_3^- from the roots to the medium was confirmed when the soybean plants were transferred from the $^{15}NO_3^-$ medium to the non-labeled NO_3^- medium (Ohyama et al. 1989b). The results suggested that the presence of three steps of efflux: first a rapid efflux (5 mgN hr^{-1}) for the initial few min, and the second efflux (0.9 mgN hr^{-1}) for about 10 min, and the third efflux (0.4 mgN hr^{-1}) continued for a several hrs. When plants were transferred from $^{15}NO_3^-$ solution to

N-free solution, the efflux was very low compared with non-labeled NO_3^- solution. This suggests that nitrate efflux system in soybean roots operate only in the presence of external NO_3^-.

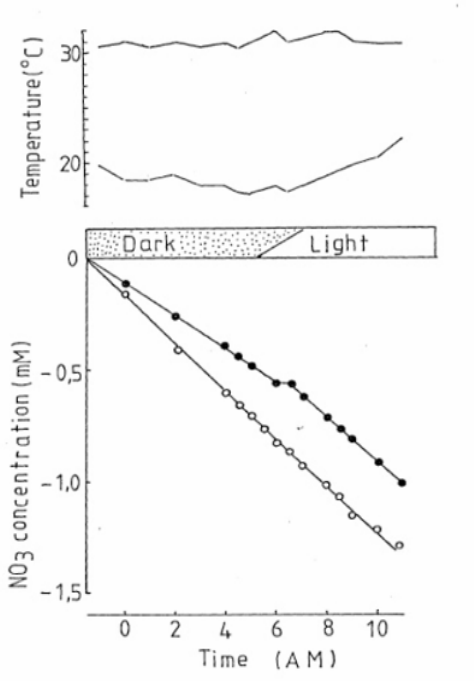


Figure 32. Diurnal changes in NO_3^- absorption rate by soybean root from culture solution under natural temperature changes (closed circle) and continuous temperature at 30 ^oC (open circle).

3. NITRATE ASSIMILATION IN PLANTS

Some part of the NO_3^- absorbed in the root cell is reduced to nitrite (NO_2^-) by nitrate reductase (NR) in cytosol, then reduced to ammonia by nitrite reductase (NiR) in plastids followed by assimilation via GS/GOGAT pathway to amino acids (Figure 25) . When a high concentration of NO_3^- is supplied, a part of NO_3^- is temporary stored in vacuoles. Some part of NO_3^- is transported cell to cell via symplast pathway and effluxed in the stele and transported via xylem with transpiration stream in the form of NO_3^-.

NR catalyzes the reaction as follows:

$$NO_3^- + NAD(P)H + H^+ \rightarrow NO_2^- + NAD(P)^+ + H_2O$$

Plant NR requires NADH or NAD(P)H witch means both NADH and NADPH as electron donor. In soybean, there are tow types of NAD(P)H-NR and one type of NADH-NR (Harper 1987).

NiR catalyzes the reaction as follows:

$$NO_2^- + 6\ Fd_{red} \rightarrow NH_4^+ + 6Fd_{ox} + 2H_2O$$

Where Fd_{red} is reduced type of ferredoxin and Fd_{ox} is oxidized type of ferredoxin.

4. COMPARISON OF NO_3^-, NO_2^- AND NH_4^+ ABSORPTION AND TRANSPORT IN SOYBEAN

Assimilation and transport of nitrate was compared with nitrite and ammonium using ^{15}N labeled compounds (Ohyama et al. 1989a). The nodulated soybean plants were treated with a culture solution containing 10 mgN L^{-1} (0.7 mM) $^{15}NO_3^-$, $^{15}NO_2^-$, or $^{15}NH_4^+$, and the assimilation and transport of N originated from these compounds was compared for 24 hr. The absorption rate of N and their partitioning patterns among roots, nodules, stems and leaves were very similar for $^{15}NO_3^-$ and $^{15}NH_4^+$ (Figure 33).

The N from both N sources was rapidly transported to the stems and leaves and readily assimilated into the protein (80% ethanol insoluble fraction). During 24 hr of ^{15}N feedings, approximately 70% of N originated from $^{15}NO_3^-$ and $^{15}NH_4^+$ was partitioned in the leaves plus stems. However, the absorption of $^{15}NO_2^-$ was about half as much as those of $^{15}NO_3^-$ and $^{15}NH_4^+$. In addition, the N originating from $^{15}NO_2^-$ was accumulated in the roots, and not readily transported to the shoots (only about 20% partitioning in leaves plus stems after 24hr). Different from soybean, ammonium uptake was twice as fast as nitrate uptake in lupin plants which were supplied with 2.8 mM NH_4NO_3 (Atwell, 1992).

After the addition of $^{15}NO_3^-$ in the solution, the asparagine concentration increased markedly, indicating that asparagine is a major assimilatory compounds of NO_3^- in soybean roots. When $^{15}NH_4^+$ was supplied in the solution, the concentration of glutamine in roots increased very rapidly in 4 hr, then asparagines concentration increased linearly in 24hr. On the other hand, when

$^{15}NO_2^-$ was supplied, glutamine level increased rapidly in the roots, but the concentration of asparagine did not.

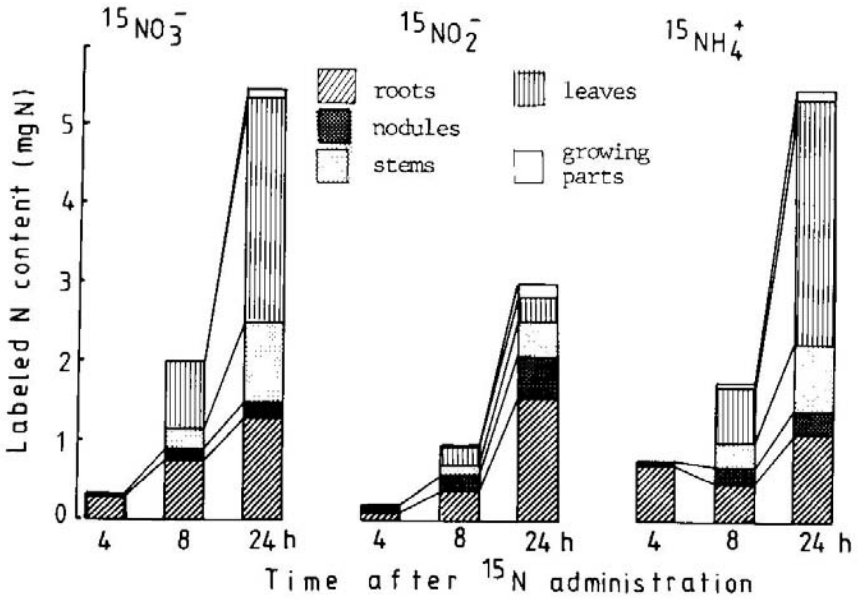


Figure 33. Comparison of N absorption and translocation in soybean plants supplied with NO_3^-,. NO_2^- or NH_4^+. From Ohyama et al. 1989a

Nitrogen assimilation and transport of the plants supplied with $^{15}NO_3^-$ was investigated by analyzing xylem sap collected from decapitated soybean plants (Ohyama et al. 1989c). The ^{15}N abundance of xylem sap increased very rapidly, about 8% of N in xylem sap collected during first 15 min originated form the N absorbed $^{15}NO_3^-$ (Figure 34). The time course of the changes in the ^{15}N abundance of xylem sap indicated that some part of absorbed NO_3^- is very rapidly transported through xylem, but, the other part may be transported slowly after once stored in the roots. In xylem sap collected at flowering stage contained about the same levels of NO_3^- and asparagine, as primary compounds of NO_3^- transport in soybean.

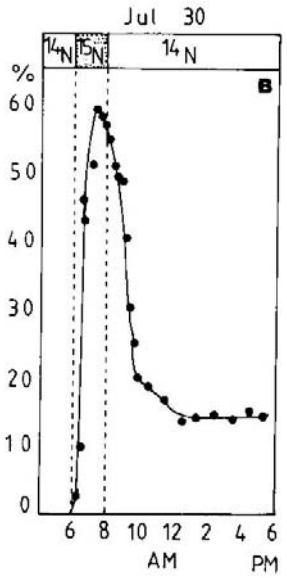


Figure 34. ^{15}N abundance (%) of xylem sap collected from the stamps of the roots supplied with $^{15}NO_3^-$ for 2 hours and chase period of 10hours. From Ohyama et al. 1989b

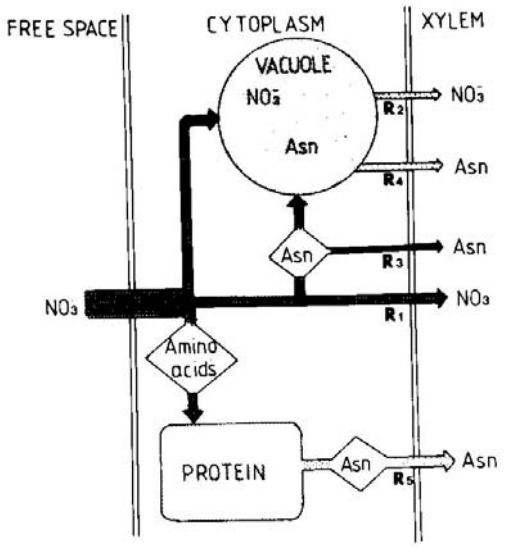


Figure 35. A model of NO_3^- absorption and metabolism in soybean roots. From Ohyama et al. 1989b

Concerning to nitrate assimilation and transport in the roots, we proposed the hypothetic scheme as shown in Figure 35 (Ohyama et al. 1989a). Some part of NO_3^- absorbed in the roots is immediately exported to the shoots, whereas another part of NO_3^- is temporarily stored in the vacuoles of root cells then gradually released to the xylem. On the other hand, some other part of NO_3^- is reduced and assimilated in the roots and synthesized in asparagine. Some part of asparagine is transported immediately after assimilation in the root cytoplasm, while another part of asparagine is once stored in vacuoles and released gradually. The degradation product of root protein may be exported as in the form of asparagine.

5. NITRATE ABSORPTION AND ASSIMILATION IN SOYBEAN NODULES

NO_3^- transport pathway into soybean nodule was investigated by tungstate and $^{15}NO_3^-$ tracers (Mizukoshi et al. 1995). There are several possible pathways of NO_3^- transport into soybean nodules. First the NO_3^- absorbed from the lower part of roots is transported through the xylem and supplied to the nodules from xylem. Second, NO_3^- is supplied by phloem from shoots, which was transferred from xylem to phloem in stems or leaves. Third, NO_3^- is directly absorbed from nodule surface. Forth, NO_3^- absorbed from the adjacent root part is transported cell to cell from root cortex to nodule cortex via the symplastic pathway. The importance of symplastic transport of C unloaded from nodule phloem and N from infected cells were suggested in soybean nodules (Brown et al., 1995).

Tungstatre (WO_4^{2-}) was used as an anion tracer, and the distribution of tungsten (W) in the roots and nodules was examined by electron probe X-ray microanalysis (EPMA). At 3 days after 1 mM WO_4^{2-} treatment in culture solution, accumulation of W in the roots cortex was observed while the W movement into root nodules was not detected. It was suggested that external anions cannot be readily transported into nodules via apoplastic pathway.

In contrast, when 1.7 mM of $^{15}NO_3^-$ was supplied to the solution for 1 day, an appreciable amount of NO_3^- and ^{15}N was detected in nodule cortex, although a little was distributed in the infected region. In another experiment, $^{15}NO_3^-$ solution was supplied to one large nodule through a cheese cloth wrapping the nodule. These results suggest that NO_3^- can be absorbed from nodule surface (epidermal cells or loosely packed lenticel cells), then it is transported from cell to cell into cortex via simplistic pathway. Arrese-Igor et al. (1998) reported that nitrate accumulation was 5 times higher in cortex than infected region of the soybean

nodules supplied with 10 mM NO_3^- for 8 days. The nitrate treatment did not cause free nitrite accumulation in nodules in 8 days.

The bacteroid has NR activity when soybean plants are cultivated without nitrate. The characteristics of nitrate respiration of isolated soybean bacteroids were reported (Ohyama and Kumazawa 1987). Under anaerobic conditions, the respiratory CO_2 evolution was very low. When 1 mM NO_3^- was added, the CO_2 evolution increased by 18 times. The CO_2 evolution associated with NO_3^- reduction was inhibited by the addition of 1 mM DNP (2,4-dinitrophenol) or 1 mM $HgCl_2$, but not by 1 mM KCN. On the other hand, aerobic respiration at pO_2=0.2 was severely depressed by 1 mM KCN and 1 mM $HgCl_2$.

Chapter 4

NITROGEN INHIBITION ON NODULE GROWTH AND NITROGEN FIXATION

1. NITRATE INHIBITION ON NODULE FORMATION AND NITROGEN FIXATION

The inhibitory effects of externally supplied N especially NO_3^- have been reviewed (Streeter 1988, Harper 1987), however the nitrate inhibition is complex and we cannot explain by a single mechanism. It has been suggested that there are multiple effects of nitrate inhibition, such as the decrease in nodule number, nodule mass, and N_2 fixation activity, as well as the acceleration of nodule senescence or disintegration (Streeter 1988, Harter 1987). In addition, nitrate inhibition of nodules is complex, because the effects of nitrate on nodule formation and growth are influenced by nitrate concentration, placement and treatment period as well as legume species (Harper and Gibson 1984, Gibson and Harper 1985, Davidson and Robson, 1986).

Nitrate inhibition is primarily host plant dependent and it is independent of nitrate metabolism of rhizobia (Gibson and Harper 1984, Carrol and Mathews 1990). Many hypothesis are proposed for the cause of nitrate inhibition of nodulation and N_2 fixation, i.e. carbohydrate deprivation in nodules (Streeter 1988, Vessy and Waterer 1992), feedback inhibition by a product of nitrate metabolism such as glutamine (Neo and Layzell 197), asparagine (Bacanambo and Harper 1996, 1997), and decreased O_2 diffusion into nodules which restricts the respiration of bacteroids (Schuller et al. 1988, Vessey et al. 1988, Gordon et al. 2002). Kanayama and Yamamoto proposed that NO formed from NO_3^- binds to Lb to make nitrosylleghemoglobin and defect the O_2 binding activity (Kanayama

and Yamamoto 1990abcd). On the other hand, Giannakis et al. (1988) suggested that nitrate metabolism does not occur in symbiotic region of soybean nodule, even when a dissimilatory NR is expressed, because of restricted access of nitrate.

It is well recognized that plant growth is affected by various environmental factors, such as temperature, moisture, photoperiod, light intensity and quality, as well as physical, chemical, and biological properties of soil. The degree of nitrate inhibition was affected by soil medium composition with vermiculite and perlite, where the proportion of solid, liquid and gas space was changed (Nishiwaki et al. 1995). The effect of planting density and nitrogen supply (0 mM and 5mM NO_3^-) was investigated at 31 days after planting (Nishiwaki et al. 1996). The higher the planting density imposed, the lower the depression of nodulation. This may be due to the rapid depletion of NO_3^- from medium. The total N concentration of leaves, stems and roots was significantly high in 5 mM NO_3^- supply compared with 0 mM NO_3^- supply, although no significant difference was observed in nodules. In 5 mM NO_3^- supply, the total N concentration as well as nitrate and total amino acids N concentration in leaves and stems decreased by increasing planting density. The sugar concentration was lower in 5 mM NO_3^- supply than 0 mM NO_3^- supply in stems and roots but not in leaves. The consumption of sugars in roots and stems may be accelerated for NO_3^- absorption and assimilation.

2. LONG-TERM EFFECT OF NITRATE SUPPLY ON NODULE FORMATION AND NITROGEN FIXATION

Local and systemic effect by nitrate on nodulation has been reported in leguminous plants. The local effect of nitrate inhibition was shown in split-root experiments where root systems had been separated into tow equivalent parts. The strong and rapid nitrate inhibition of nodule growth and N_2 fixation activity is restricted in the nodules attached to the root portions that are in direct contact with nitrate; and no or milder inhibition is induced in the other part of the root system receiving no nitrate (Tanaka et al., 1985). However, some systemic inhibition of nitrate on nodulation and nitrogen fixation has also been observed with a high concentration of nitrate in clover (Silsbury et al. 1986).

We investigated the local and systemic effect of continuous NO_3^- supply by using horizontal sprit root system in two layered pot system, where the lower part of roots were supplied with culture solution containing 1mM NO_3^- in the lower pot, and the upper roots were in the vermiculite medium with N-free culture solution in the upper pot (Ohyama et al. 1993a). The soybean plants (cv. Williams

and Norin No.2) were cultivated with 0 mM or 1 mM NO_3 solution in the lower pot, and harvested at maturing (R7) stage. In this stage, there are no nodules remained in the lower part of roots. The dry weight of shoot and upper part of roots were almost the same between 0 mM and 1 mM NO_3 supply, but 1 mM NO_3 supply decreased the dry weight of nodules attached in the upper part of roots in both varieties. This result indicates that continuous long term supply of NO_3 may impose systemic inhibition of nodulation or acceleration of nodule senescence in soybean plants.

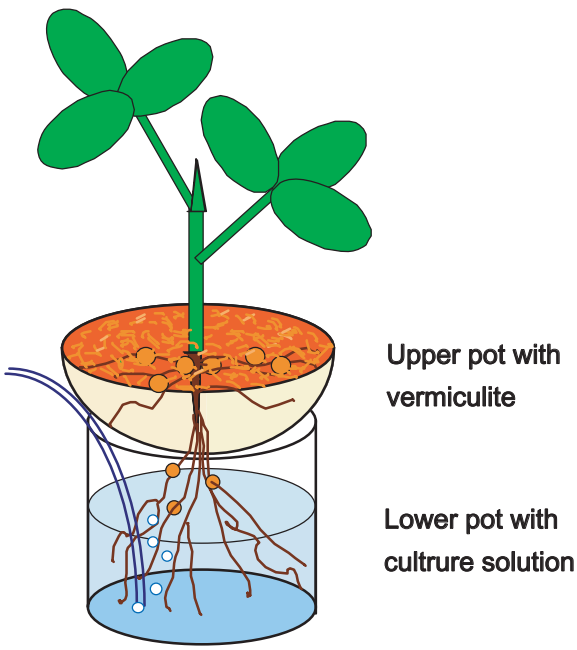


Figure 36.Vertical split root expeiment using a two-layered pot system. From Yashima et al. 2003

Systemic and local effects of long-term application of nitrate on nodule growth and N_2 fixation in soybean plants were more precisely investigated using tow layered pot system (Figure 36, 37). Four treatments were imposed i.e. 0/0, 0/5, 5/0 and 5/5, with the 0 mM or 5 mM NO_3^- treatment in upper pot/ lower pot, respectively. The plants were harvested at the initial flowering (R1) stage and pod setting (R4) stage, and the effect of nitrate placement on nodule number, nodule growth, and N_2 fixation was elucidated (Yashima et al. 2003). As shown in Figure 38, the development of the root system in the lower pots was quite different

between 0 and 5 mM NO_3^- in the lower pot. The root length was longer in 0 mM treatment in lower pot (0/0, 5/0), but a bunch of short lateral roots was formed in the solution with 5 mM NO_3^- in lower pot (0/5, 5/5). In the lower pot where the nodules were in direct contact with 5 mM NO_3^-, the inhibition on the nodule number, nodule size and N_2 fixation was conspicuous. Systemic and local effect on nodule number per plant did not occur in the upper nodules in vermiculite. On the other hand, systemic inhibition on the nodule dry weight and N_2 fixation activity in the upper pot was apparent. The 5/5 treatment depressed the nodule growth and nitrogen fixation activity in the upper nodules. Nitrate accumulation was observed only in the part of roots and nodules in direct contact with 5 mM NO_3^- either in the upper or lower pot. The concentration of total amino acids was higher in the lower roots in 0/5 treatment than 0/0 treatment, however, that was almost the same level in the roots and nodules of the upper part both at R1 and R4 stage.

Figure 37. A photograph of soybean plants cultivated with two-layered pot system.

The soluble sugar concentration in the lower roots in 0/5 treatment was lower than that in the 0/0 treatment. The similar trend was observed in the upper roots of 0/5 treatment, suggesting that the absorption of NO_3 from the lower roots decrease sugar concentration in both lower roots in direct contact with nitrate, and the upper roots not contact with NO_3^-.

Figure 38. A photograph of soybean roots cultivated with two-layered pot system with 0/0, 0/5, 5/5, 5/0 treatments (from left to right). From Yashima et al. 2003

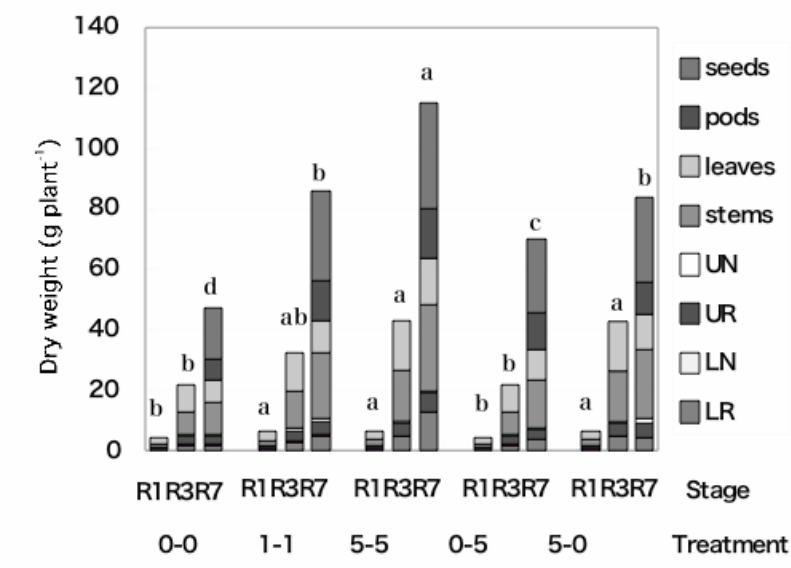


Figure 39. Effect of nitrate treatment on the dry weight of each part of soyban plants at R1, R3 and R7 stages. 0-0, 1-1, 5-5, 0-5, 5-0 indicate nitrate concentration (mM) in the lower pots from planting to R1, and R1 to R7. From Yashima et al. 2005.

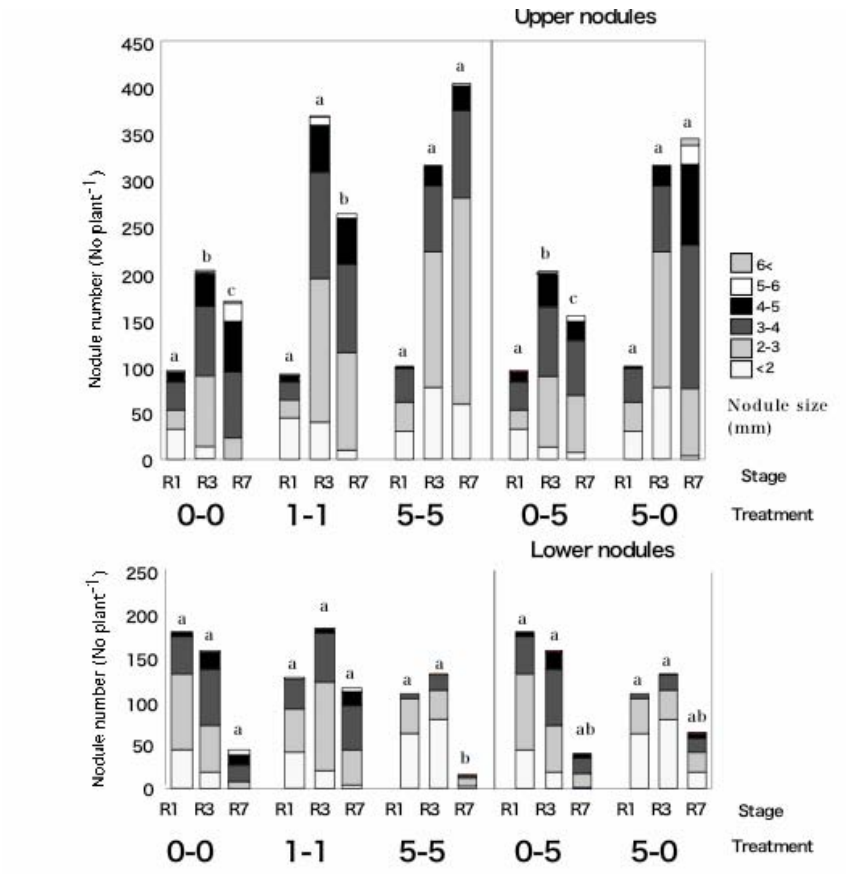


Figure 40. Effect of nitrate treatment on the number of soyban nodules at R1, R3 and R7 stages. 0-0, 1-1, 5-5, 0-5, 5-0 indicate nitrate concentration (mM) in the lower pots from planting to R1, and R1 to R7. From Yashima et al. 2005

Long-term effect of NO_3^- application from the lower part of roots on the nodulation of the upper part of roots was further investigated in relation to concentration and treatment period (Yashima et al. 2005). The solution with 0 mM, 1mM or 5 mM NO_3^- was supplied from transplanting to two-layered pot system at 14 days after planting to R7 stage. Five treatments were imposed that 0-0 treatment (continuous 0 mM NO_3^-), 1-1 treatment (continuous 1 mM NO_3^-), 5-5 treament (continuous 5 mM NO_3^-), 0-5 treatment (0 mM until R3 then 5 mM NO_3^-), and 5-0 treatment (5 mM until R3 then 0 mM NO_3^-). Total plant dry weight and seed dry weight was highest in 5-5 treatment, intermediate in the 1-1, 5-0, 0-5 treatments, and lowest in the 0-0 treatment (Figure 39). Figure 40 shows the

nodule number per plant classified with nodule diameter. The nitrate supply in the lower pot increased the total nodule number in the upper roots, although decreased the number of nodules in the lower roots. The value of the nodule dry weight per plant (Figure 41) and N_2 fixation activity (acetylene reduction activity: ARA) per plant (Figure 42) and ARA per nodule dry weight (Figure 43) were lowest in the 5-5 treatment. Interestingly, the nodule dry weight in the upper roots was highest in the plants with 1-1 treatment and exceeded the 0-0 treatment (Figure 40). The acetylene reduction activity per plant of the upper nodules at R3 stage was also highest in the 1-1 treatment (Figure 42). This was due to the increase in nodule dry weight and not to ARA per dry weight of nodules. These results indicated that continuous supply of low concentration of NO_3^- from the lower roots does not inhibit the nodule growth and N_2 fixation activity, but it can promote nodulation and N_2 fixation. Figure 44 shows the example of soybean root system cultivated with continuous supply of 1 mM nitrate at R3 stage. Nodulation was enhanced in the upper roots by supplying 1mM NO_3^- from the lower roots.

Withdrawal of 5 mM NO_3^- after R3 stage in 5-0 treatment markedly enhanced nodule growth (Figure 40) and acetylene reduction activity at R7 stage (Figure 42) when the values of both parameters decreased in the other treatments. The nitrate concentration of the nodules attached to the upper roots was very low, including continuous supply of high concentration of NO_3^- in 5-5 treatment. This result indicated that the inhibitory effect of 5 mM NO_3^-, or promotive effect of 1 mM NO_3^- was not directly controlled by nitrate itself, but was mediated through some systemic regulation such as sucrose supply.

3. Short-Term Effect of Nitrate Supply on Nodule Formation and Nitrogen Fixation

Concerning to direct effect of NO_3^- on nodule growth and N_2 fixation activity, Fujikake et al. recently discovered quick and reversible inhibition of nodule growth by nitrate (Fujikake et al. 2002. 2003b). The upper part of nodulated soybean root hydroponically cultured in a glass bottle was monitored using a computer microscope under controlled environmental conditions, and the diameter of individual nodules was measured from 10-24 days after planting (Figure 45). The diameter of a nodule attached to the primary roots increased from 1 mm to 6 mm for 2 weeks under N free conditions (Figure 45a). The increase in nodule diameter was almost completely stopped after 1 d of supplying 5 mM NO_3^-

(Figure 45b). However, nodule growth quickly returned to the normal growth rate following withdrawal of NO_3^- from the solution (Figure 45c).

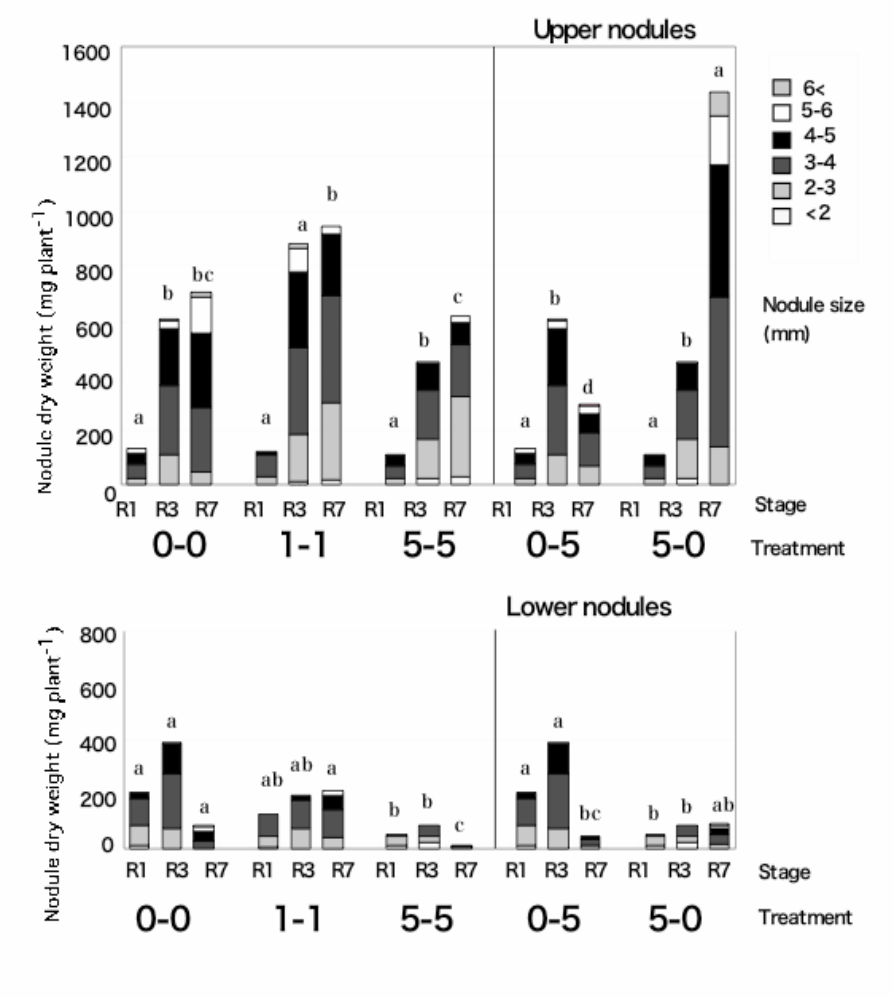


Figure 41. Effect of nitrate treatment on the dry weight of soyban nodules at R1, R3 and R7 stages. 0-0, 1-1, 5-5, 0-5, 5-0 indicate nitrate concentration (mM) in the lower pots from planting to R1, and R1 to R7. From Yashima et al. 2005

The reversible depression of nodule growth by NO_3^- was similar to the restriction of the photoassimilate supply by continuous dark conditions for 2days followed by normal light/dark conditions (Fujikake et al. 2003b). In addition, the

inhibitory effect of nitrate was partially alleviated by the addition of 3% sucrose to the culture solution (Fujikake et al. 2003b).

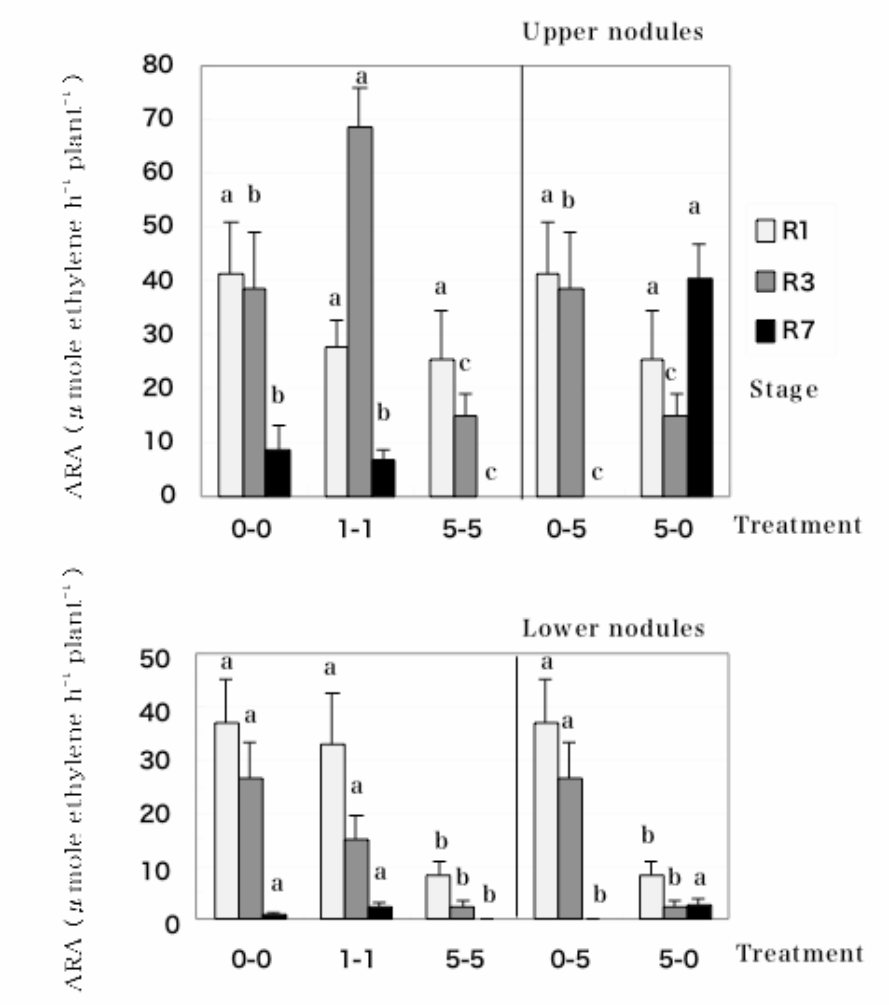


Figure 42. Effect of nitrate treatment on ARA per plant of soyban nodules at R1, R3 and R7 stages. 0-0, 1-1, 5-5, 0-5, 5-0 indicate nitrate concentration (mM) in the lower pots from planting to R1, and R1 to R7. From Yashima et al. 2005

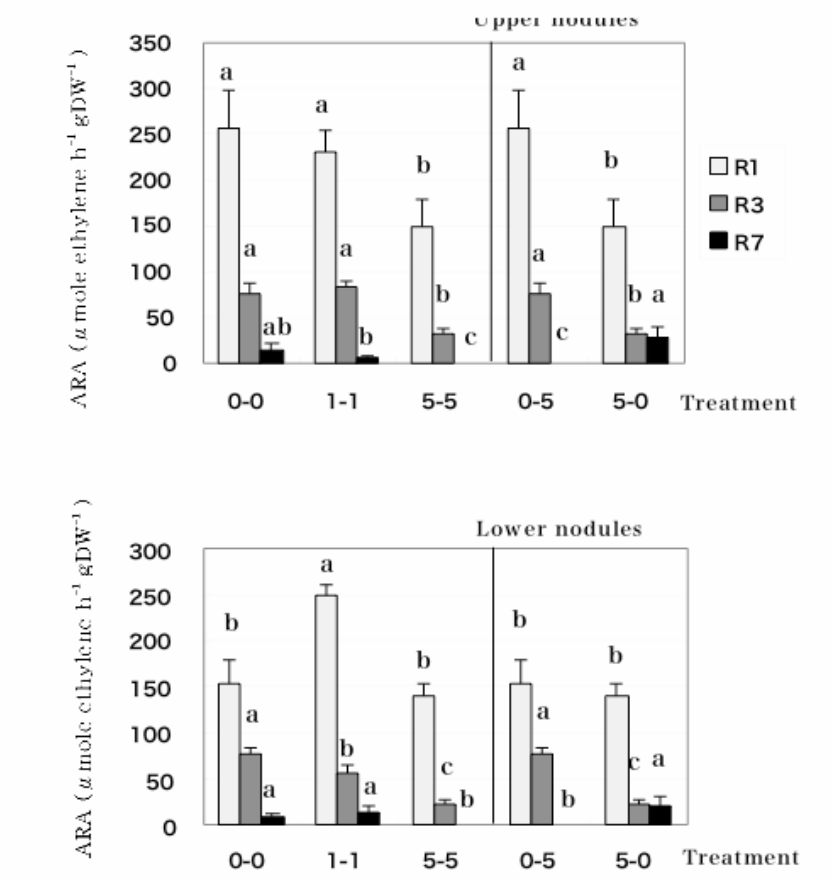


Figure 43. Effect of nitrate treatment on ARA of soyban nodules per g DW at R1, R3 and R7 stages. 0-0, 1-1, 5-5, 0-5, 5-0 indicate nitrate concentration (mM) in the lower pots from planting to R1, and R1 to R7. From Yashima et al. 2005.

Split-root system was made by cutting the primary root of soybean seedling at 24 days after planting. The split roots were supplied with solution containing with 0 mM or 5 mM NO_3^- for 3 days from 27-29 days after planting. The positron emitting radioisotope $^{11}CO_2$ was supplied to the first trifoliolate leaves of 29 days after planting for 10 min. The movement of ^{11}C was monitored by positoron emitting tracer imaging system (PETIS). Compared with the root system supplied with 0 mM NO_3^- and 5 mM NO_3^-, the transport rate of ^{11}C was faster in the roots supplied with 5 mM NO_3^- than those in 0 mM NO_3^- (Figure 46). Quantitative evaluation was conducted using ^{14}C as a tracer. By supplying 5 mM NO_3^-, the ^{14}C

partitioning to nodule decreased from 9.1 % to 4.3%, while that to the roots increased from 5.2 % to 9.1% (Figure 47).

Figure 44. Effect of nitrate treatment on nodule formation in the upper (left) and lower parts of soybean roots cultivated with two-layered pot at R3 stage. 1 mM nitrate was continuously supplied in the lower pots. From Yashima et al. 2005

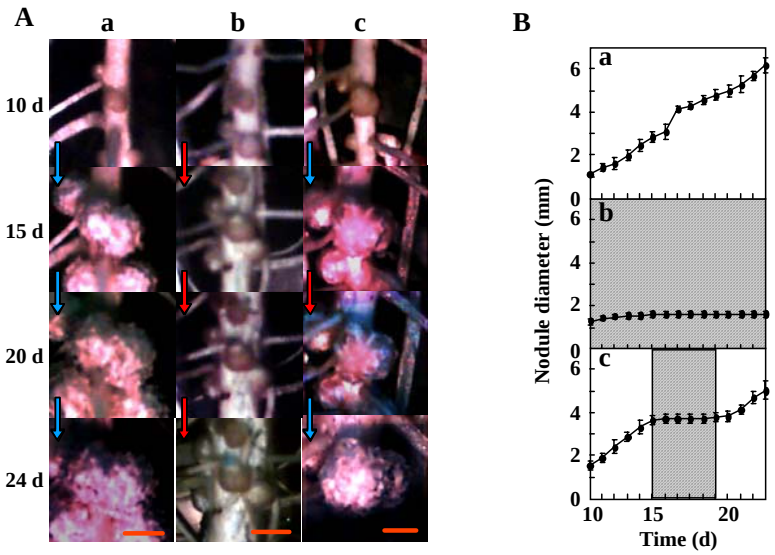


Figure 45. Growth response of soybean nodules to 0 mM (blue arrows) or 5 mM nitrate (red arrows) nitrate application in the culture solution. (a) 0 mM nitrate, (b) 5 mM nitrate, (c) 0, 5, 0 mM nitrate. From Fujikake et al. 2003

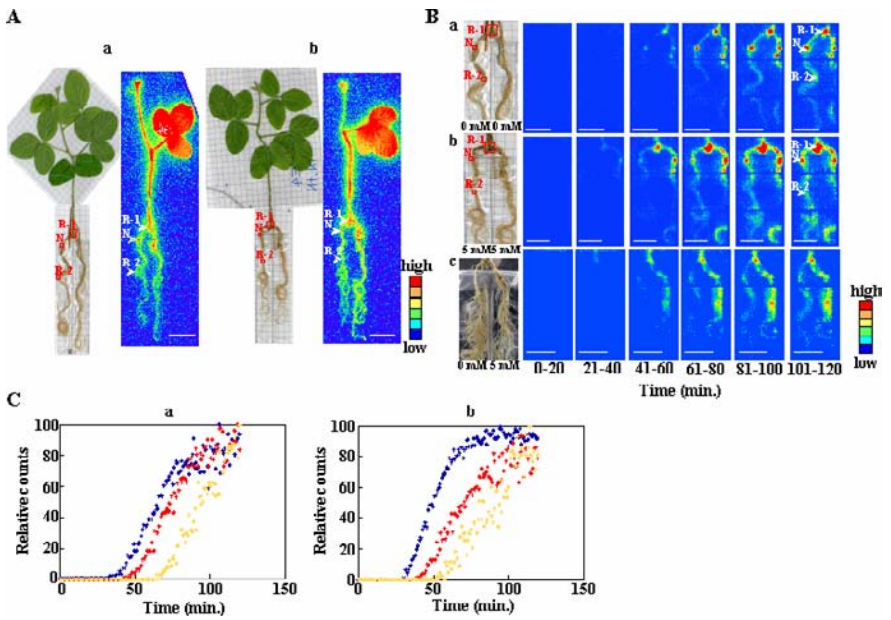


Figure 46. ^{11}C traaanslocation in the split root systems of soybean plants. (A) Real time images of the distribution of ^{11}C in soybean using Imaging Plant (BAS-1500). (B) Time course for the accumulation of radioacrivity as monitored by PETIS (Positoron emitting tracer imaging system), (C) The accumulation of radioactivity for the various point of roots. From Fujikake et al. 2003

These results indicate that the decrease in photoassimilate supply to nodules may be involved in the quick and reversible nitrate inhibition of soybean nodule growth and N_2 fixation activity (Figure 48). The decrease in starch concentration in nodules (Vessey et al. 1988, Gordon et al. 2002) and the down-regulation of sucrose synthase transcript within 1 day of nitrate treatment (Gordon et al. 2002) may imply that NO_3^- reduces photoassimilate flow into nodules and sucrose utilization in nodules.

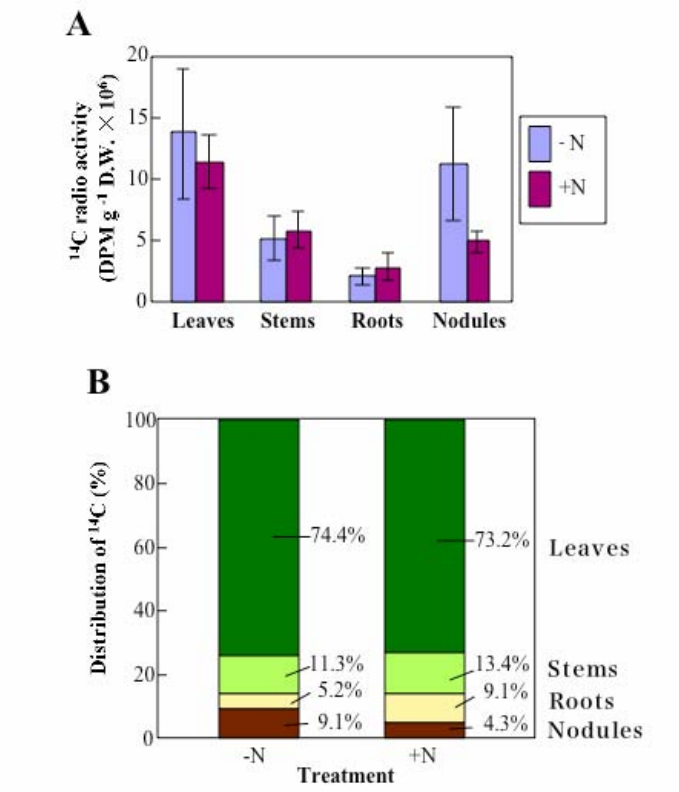


Figure 47. Partitioning of ^{14}C labeled photoassimilate in soybean plants supplied with 0 mM (-N) or 5 mM (+N) nitrate. From Fujikake et al. 2003

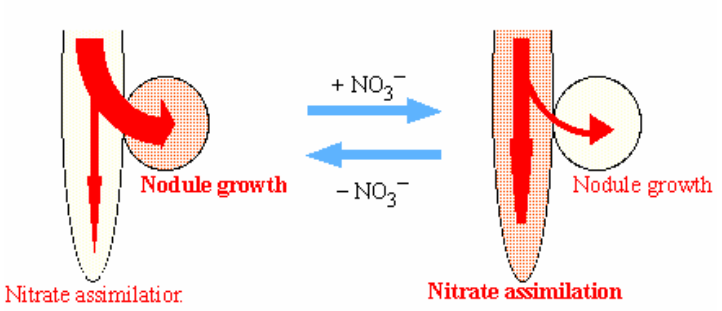


Figure 48. The mechanism of quick and reversible nodule growth and nitrogen fixation activity by short term effect of nitrate.

Chapter 5

NITROGEN ASSIMILATION AND NITRATE TORLERANCE OF HYPERNODULATION MUTANTS OF SOYBEAN

1. HYPERNODULATION MUTANT OF SOYBEAN

Hypernodulation or supernodulation (in synonym) mutant lines of soybeans has been selected from several different cultivars of soybean (Akao and Kouchi 1992, Carroll et al., 1985, Gremaud and Harper 1989). A genetic defect in autoregulatory control of nodulation causes profuse nodulation than wild type (Figure 49). By grafting experiment, the control of nodulation is based on the communication between roots and shoots. Nodulation trait depends on shoot genotype and not on root genotype (Figure 50). In wild type parents, some shoot derived signal (autoregulation signal) arrests nodule primordia (Gerahty et al 1992) and suppresses nodule development in response to some signals (infection signal) derived from nodulated roots after infection. Sato et al. (1997a) reported that rooted single leaf of soybean retains autoregulation traits as shown in Figure 51. Only a few nodules are formed in Williams parent rooted leaf, but almost 50 nodules attached in the hypernodulation mutant line NOD1-3. This rooted single leaf system does not have stems and shoot apical meristems, therefore, autoregulation control of nodule number is controlled by mature leaves without stems and shoot apical meristems.

Figure 49. A photograph of nodulated roots of Williams (left) and its hypernodulation mutant line NOD1-3 (right) cultivated with N free solution.

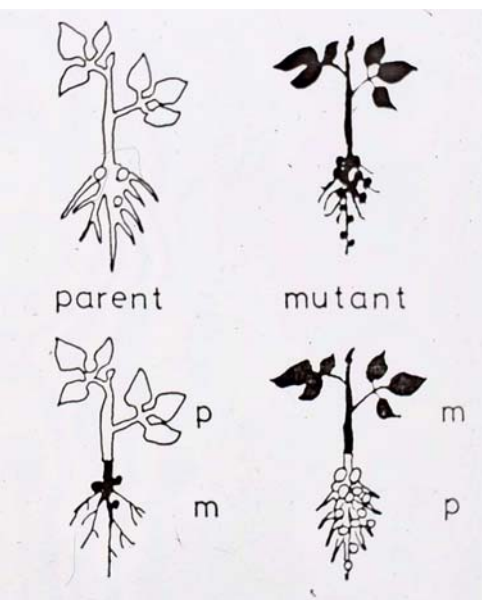


Figure 50. Evidence of shoot control of autoregulation by grafting experiment between hypernodulation mutant and its parent. m: mutanti, p: parent.

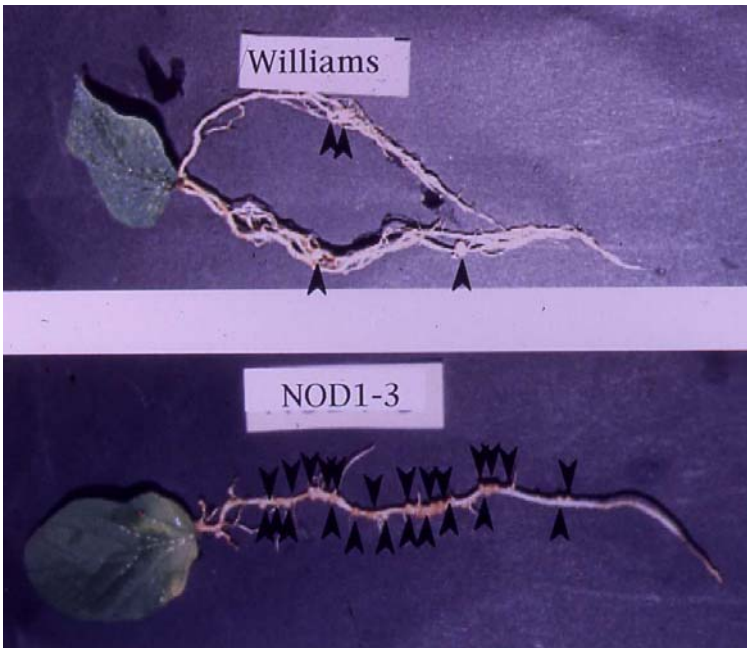


Figure 51. A photograph of nodulation of rooted single leaf of Williams (up) and its hypernodulation mutant line NOD1-3 (down) cultivated with N free conditions. From Sato et al. 1997

2. PARTIALLY NITRATE TOLERANT OF HYPERNODULATION MUTANT

In spite of profuse nodulation, the root and shoot growth is usually inferior in hypernodulation mutant lines compared with wild type either cultivated with 0 mM or with 5 mM nitrate (Figure 52).

NO_3^-	**0 mM**		**5 mM**	
Line	**Williams**	**NOD1-3**	**Wiliams**	**NOD1-3**

Figure 53 shows the dry weight and the distribution of dry matter of 18 days after planting followed by 4 days of 0 mM or 5 mM NO_3^- treatment (Fujikake et al 2003b). Total dry weight of Williams with 0 mM NO_3^- was about 450 mg/plant, and that of NOD1-3 was about 260 mg/plant. However, the nodule dry weight of NOD1-3 was about 50 mg and almost twice as large as that of Williams. By

suppling 5 mM NO_3^-, the dry weight of nodules decreased in both lines, while the decrease in Williams was more significant than that in NOD1-3. All the hypernodulation and supernodulation mutant lines have partially tolerant to NO_3^-. The supernodulation line first reported was named as “nts”, which means “nitrate-tolerant symbiosis” mutant (Carroll et al. 1985).

Figure 52. A photograph of root system of Williams and its hypernodulation mutant line NOD1-3 cultivated in a pot with 0 mM or 5 mM NO_3^- conditions. From Sato et al. 1999

Figure 54 shows the changes in the nodule diameter in Williams and NOD1-3 during 4 days of 0 mM and 5 mM NO_3^- treatment (Fujikake et al. 2003a). At 14 days after planting, the average nodule diameter of Williams was about 2.2 mm and that of NOD1-3 was about 2.0 mm. The average nodule diameter of Williams increased about 1.0 mm for 4 days, whereas the increase was about 0.6 mm in NOD 1-3 under the 3 mM NO_3^- conditions. The number of nodules are higher in NOD1-3 but nodule growth was slow compared with nodules of Williams. The nodule growth almost stopped after one day of 5 mM NO_3^- in Williams, while the nodule of NOD1-3 continued to grow until 2 days after 5 mM NO_3^- treatment. After 4 days of 5 mM nitrate treatment, the nodule dry weight decreased by 22% in NOD1-3 and by 58% in Williams, respectively. The 5 mM nitrate treatment decreased the ARA in NOD1-3 by 60% per plant and by 50% per nodule g DW and these parameters were less sensitive to the treatment than those in Williams in

which the inhibition rate was 90% per plant and 80% per nodule dry weight. A whole shoot of Williams and NOD1-3 plants were exposed to $^{14}CO_2$ for 120 min followed by 0 or 5 mM nitrate treatment for 2 days, and the partitioning of ^{14}C radioactivity between nodules and roots were 63% and 37% in Williams and 89% and 11% in NOD1-3. Under the 5 mM nitrate conditions, the percentage of distribution of ^{14}C between the nodule and roots changed to 14% and 86% in Williams, and 39 and 61% in NOD1-3 , respectively. These results indicated that the hypernodulation mutant NOD1-3 supplied a larger amount of photoassimilate to the nodules than to the roots under nitrogen free conditions, and that the nitrate depression of photoassimilate transport to the nodules was less sensitive than that of the parent line. Hansen et al. (1992) compared Bragg and the supernodulating mutant nts1007 with short-term (3 days) supply of 4 mM nitrate. Nodule respiratory activity of Bragg was reduced by nitrate to 27% of control, but that of nts1007 was 56%.

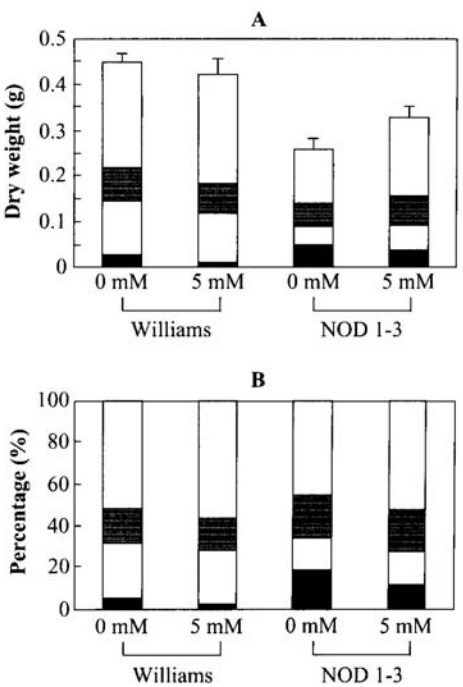


Figure 53. Comparison of dry weight and the distribution of Williams and its hypernodulation mutant line NOD1-3 cultivated in a pot with 0 mM or 5 mM NO_3^- conditions. Simbols in bar is nodules, roots, stems and leaves from down to up. From Fujikake et al. 2003

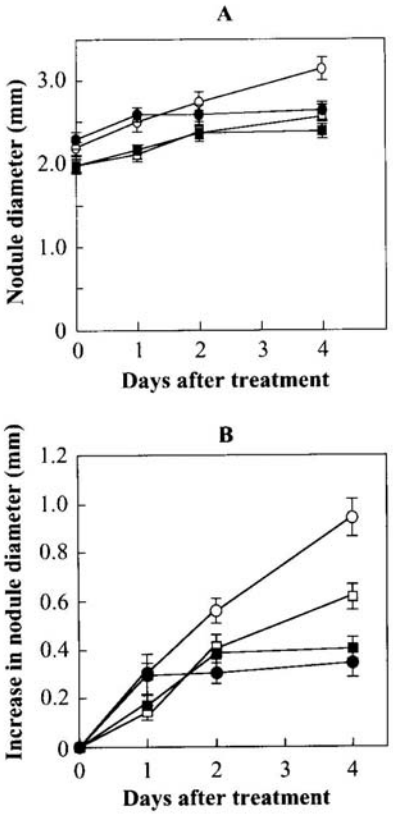


Figure 54. Changes in nodule diameter of Williams (circle) and NOD1-3 (square) cultivated with 0 mM (open) or 5 mM (closed) NO3- conditions. From Fujikake et al. 2003a

3. COMPARISON OF THE ASSIMILATION OF $^{15}N_2$ AND $^{15}NO_3^-$ BETWEEN HYPERNODULATION MUTANT AND PARENT

Assimilation of $^{15}N_2$ and $^{15}NO_3^-$ was compared among hypernodulation mutant lines, NOD1-3, NOD2-4, and NOD3-7 and the parent Williams (Ohyama et al. 1993b). Soybean seeds were inculated at planting and transplanted at day 7 to nutrient solution with 1 mM urea or 5 mM NO_3^-. At 25 days after planting, separate plants were exposed to $^{15}N_2$ or $^{15}NO_3^-$ from 3 to 48 hrs to evaluate N_2 fixation and NO_3^- assimilation. Plant growth was less for hypernodulation mutant lines than for Williams with both NO_3^- or urea nutrition. With urea grown plants the total mg ^{15}N fixed per plant for 24 h was 1.18 (Williams), 1.40 (NOD1-3),

1.07 (NOD2-4) and 0.80 (NOD3-7) (Figure 55). Distribution patterns of ^{15}N among organs were very similar among lines after a 24 h $^{15}N_2$ fixation period; approximately 40% in nodules, 12% in roots, 14% in stem, 34% in leaves. The 5 mM NO_3^- treatment resulted in a 95 to 97% decrease in nodule mass and $^{15}N_2$ fixation by Williams, while the three mutant lines retained 30 to 40% of the nodule mass and 17 to 19% of the $^{15}N_2$ fixation of respective urea grown controls. The hypernodulation mutant lines, which had restricted root growth, absorbed less $^{15}NO_3^-$ than Williams. The major part of ^{15}N from $^{15}N_2$ fixation was incorporated into insoluble fraction after 24 h, however, a larger part of ^{15}N from $^{15}NO_3^-$ retained in the soluble fraction of all plant parts through 24 h (Figure 56). These results confirmed that nodule development is less sensitive to external NO_3^- in mutant lines than in the Williams parent, and subsequent N metabolism and distribution within the plant was not different among lines. The partial tolerant of nodulation for nitrate may be due to less NO_3^- absorption activity and smaller roots in NOD lines.

The effects of decapitation (shoot removal) treatment on $^{15}N_2$ fixation, transport and assimilation were determined in NOD 1-3 and the parent Williams (Ohyama and Harper 1991). The root systems of intact plants were exposed to $^{15}N_2$ and the shoots from half of the plants were removed 1 h after the onset of $^{15}N_2$ exposure. Decapitated and intact plants were harvested at 2 h after decapitation. N_2 fixation was markedly depressed by the decapitation treatment and the amount of ^{15}N in soluble and insoluble N in the nodules was decreased. Simultaneously the concentration of amino-N, ureide-N and soluble carbohydrate in the nodules also declined following shoot removal. The response to decapitation was very similar between NOD1-3 and Williams. It is suggested that the rapid decline in N_2 fixation following decapitation was primary due to the interruption of the carbohydrate supply from shoot.

4. LEGHEMOGOLOBIN COMPONENTS IN HYPERNODULATION MUTANT AND PARENT

Leghemoglobin (Lb) plays crucial role in N_2 fixation of leguminous nodules by facilitating O_2 supply to the bacteroids. There are four major components of Lb in soybean nodules, Lba, Lbc1, Lbc2, and Lbc3, and different roles among components are suggested (Fuchsman et al. 1976), because Lba has higher affinity for O_2 than has Lbc. The concentration of Lba and Lbc were separated by Native PAGE (Nishiwaki and Ohyama 1995) or all the four components Lba, Lbc1, Lbc2,

and Lbc3 were separately determined by capillary electrophoresis (Figure 57) (Sato et al. 1997b).

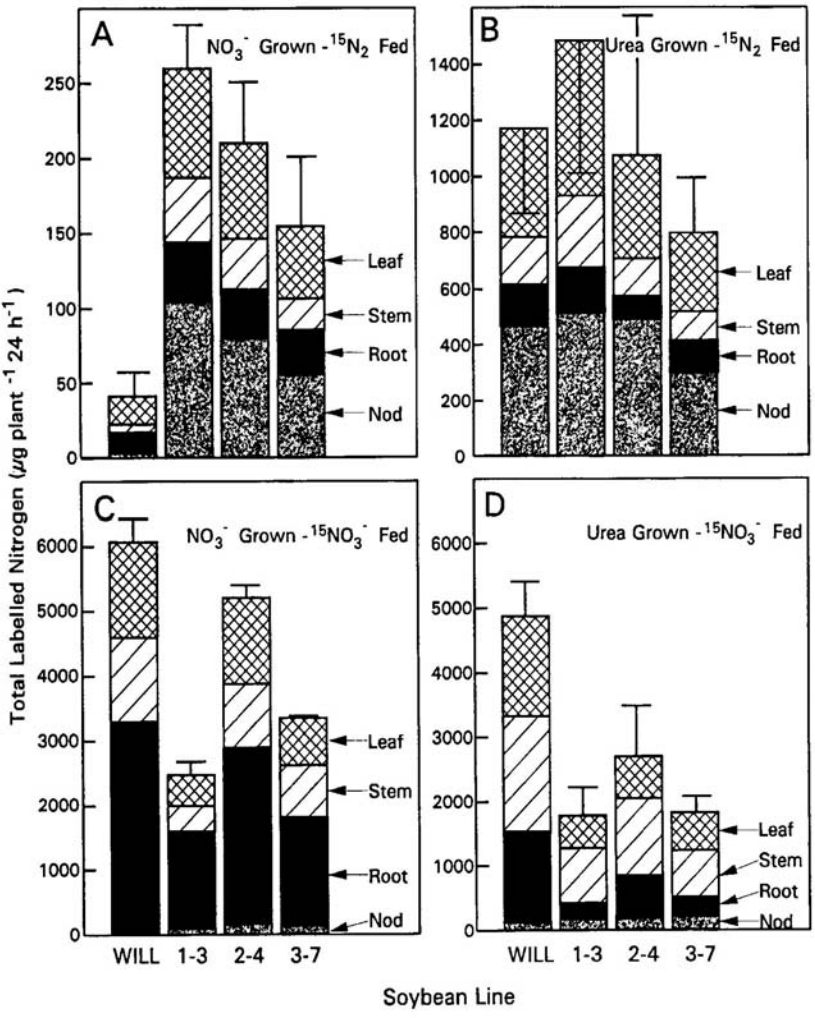


Figure 55. Total accumulation of ^{15}N labeled nitrogen in each part of Willams and the hypernodulation mutant lines NOD1-3, NOD2-4 and NOD3-7. From Ohyama et al. 1993

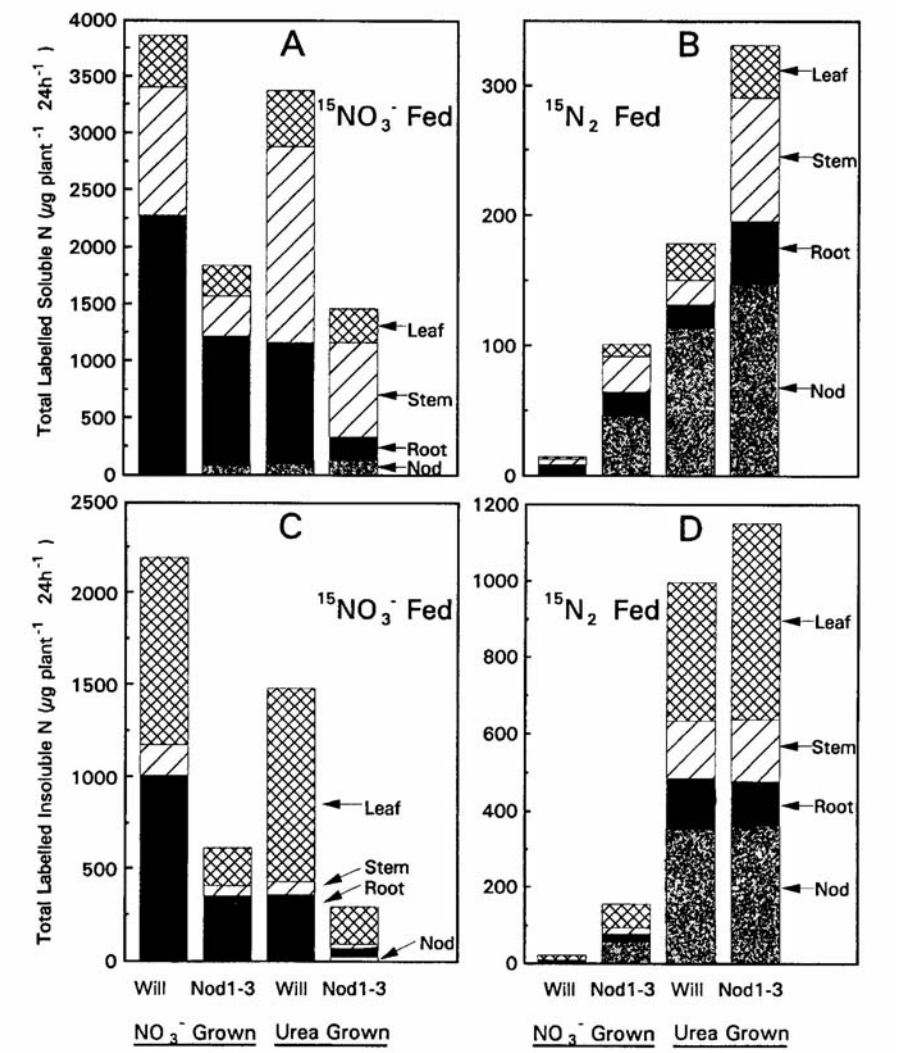


Figure 56. Accumulation of 80% ethanol soluble and insoluble ^{15}N labeled nitrogen in each part of Willams and the hypernodulation mutant line NOD1-3. From Ohyama et al. 1993

The concentration and component ratios in hypernodulation mutant NOD1-3, NOD2-4, and NOD3-7 from Williams parent, and in En6500 from Enrei parent were compared in relation to their nodulation characteristics. Three mutants (NOD1-3, NOD4 and En6500) were controlled by a single recessive allele *rj*$_7$, but NOD2-4 was non-allelic mutant to them (Vuong et al. 1996). Plants were

hydroponicully cultivated in N free solution, and the nodules were separated by size. Concentration and composition of Lb components in the same size nodules were analyzed by gel-electrophoresis and capillary electrophoresis. In all NOD mutants Lb concentration was about 70% of that in the parent Williams, irrespective of nodule size and growth stages. In the hypernodulation mutant En6500, the total Lb concentration was only 25% of that in the parent Enrei, irrespective of nodule size. In Enrei, relative compositions of Lba, Lbc1, Lbc2 and Lbc3 were 36, 26, 18 and 17%, respectively, and very stable irrespective of nodule size. EN 6500 had relatively equal amounts of each component in which the relative compositions of Lba, Lbc1, Lbc2 and Lbc3 were 30, 22, 22 and 26%. The concentration of Lbc forms in nodules were decreased by addition of nitrate to Enrei plants, but not to En6500. When the nodule morphology was compared among hypernodulation mutant lines and parent lines, we noticed that mutant line had thick cortical regions relative to the comparable parent nodules (Figure 10). The relative volume of symbiotic regions was about 50-60% of total nodule volume, but it accounted for only 40-50% in NOD mutants.

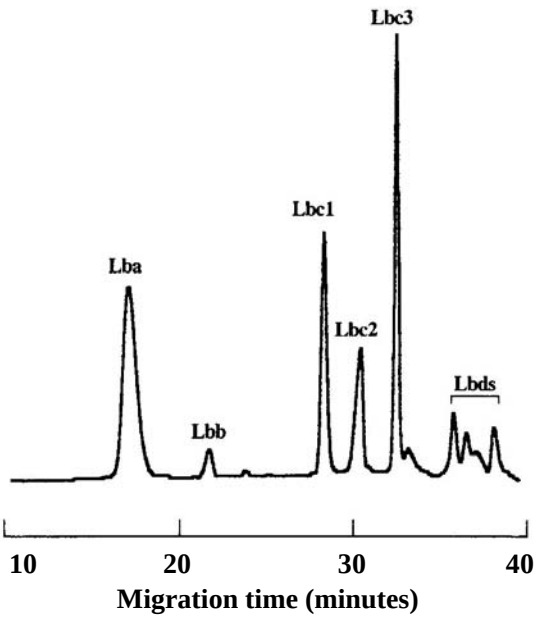


Figure 57. Analysis of leghemoglobin components by capillary electrophoresis From Sato et al. 2001

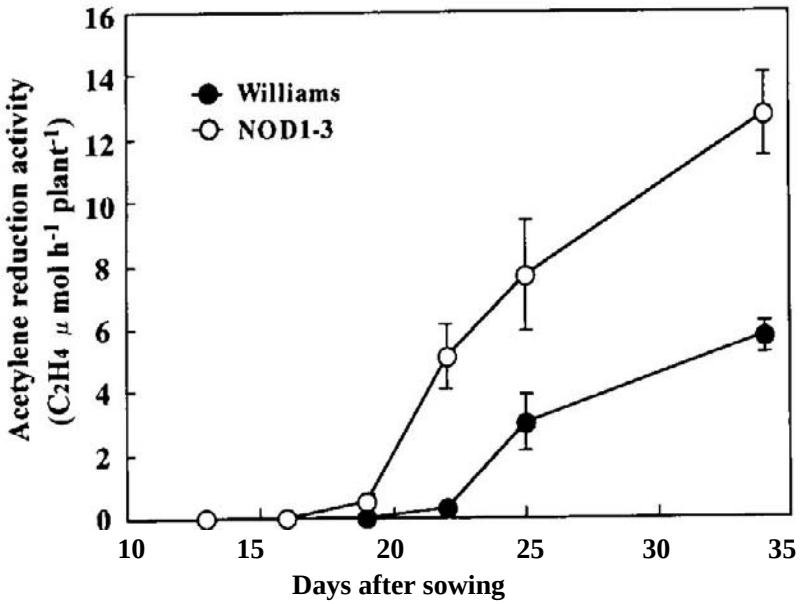


Figure 58. Changes in acetylene reduction activity per plant of Williams (closed circle) and NOD1-3 (open circle). From Sato et al. 2001

Sato et al. (2001) investigated the changes in four leghemoglobin components in nodules of NOD1-3 and its parent in the early nodule developmental stage. The hydroponically grown NOD1-3 and Williams were periodically sampled. All the visible nodules were collected from the roots and then the four Lb components in the largest nodules were analyzed by capillary electrophoresis. In NOD 1-3 nodule development was faster than those of Williams. Acetylene reduction activity was detected at 19 days after planting in NOD1-3 and at 22 days after planting in Williams (Figure 58). In addition the Lbs were initially detected at 19 days after planting, a few days earlier than in Williams at 22 days after planting (Figure 59). The Lbcs (Lbc1, Lbc2 and Lbc3) were the main component at the earliest nodule growth stage, and the relative proportion of Lba increased with nodule growth in both NOD 1-3 and Williams (Figure 60).

The hypernodulation soybean mutant lines (NOD1-3, NOD2-4, NOD3-7) and the parent Williams and mutant line En6500 and the parent Enrei were cultivated in a sandy dune field in Niigata, and the nodules and root bleeding xylem sap were analyzed at 50, 70, 90 and 120 days after planting (Sato et al 1998). The number of nodules of the hypernodulation mutant lines was about two to three times higher than that of the parent lines irrespective of sampling date. The

concentration of Lb components was measured by capillary electrophoresis. The concentration of Lb components in the hypernodulation mutant lines tended to be lower than in the parents, but the component ratios were not different between mutants and the parents.

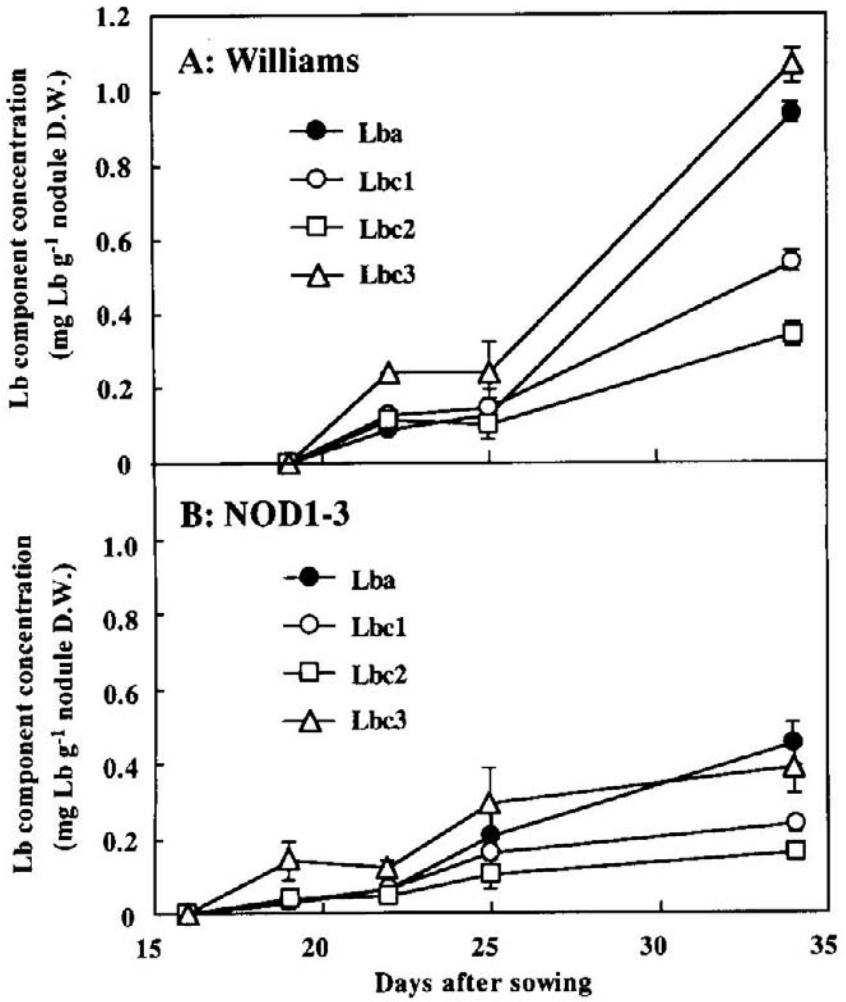


Figure 59. Changes in leghemoglobin components in nodules of Williams (up) and NOD1-3 (down) From Sato et al. 2001

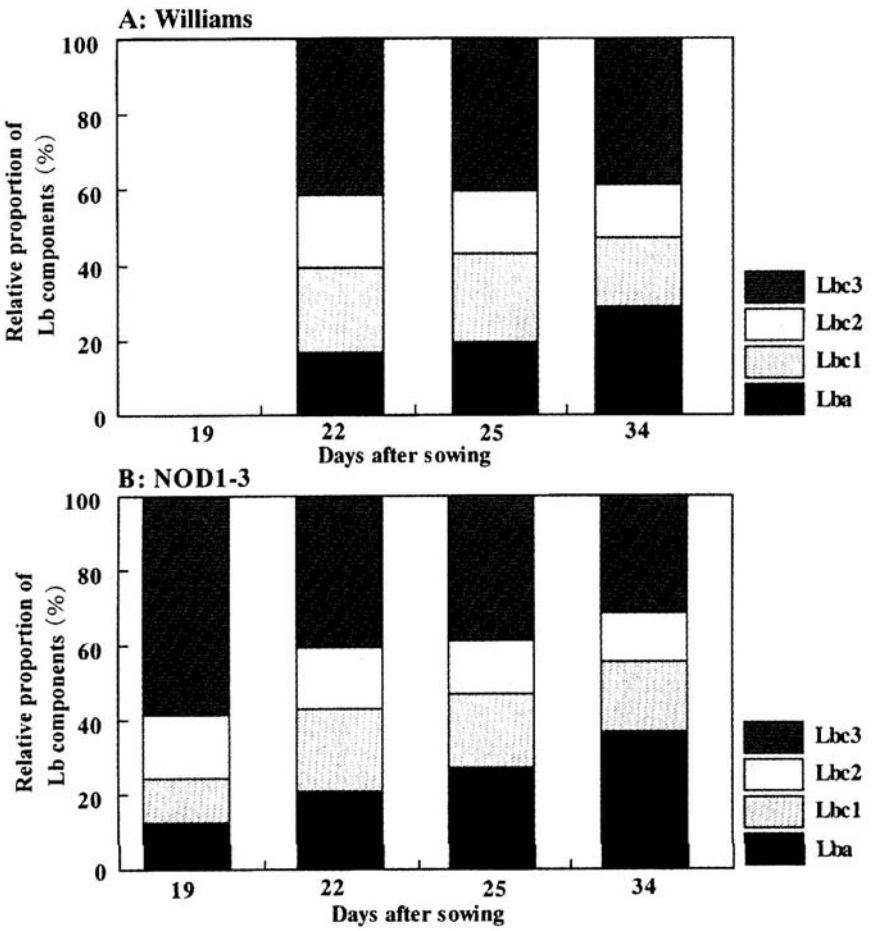


Figure 60. Changes in propotion of leghemoglobin components in nodules of Williams (up) and NOD1-3 (down). From Sato et al. 2001

5. Comparison of Nodulation and Xylem Sap Composition between Hypernodulation and Parent Cultivated in the Field

The concentrations of major nitrogen compounds (nitrate, allantoin, allantoic acid, and asparagine) in the xylem sap were also measured by capillary electrophoresis (Sato et al 1998). Warren and Adams (2000) also reported

determination of major amino acids and sugars by capillary electrophoresis. The concentration of ureide and nitrate in xylem sap decreased with the plant age, but the asparagine concentration increased in the later stage. The concentration of ureides and asparagine were relatively higher, and the nitrate concentration was lower in the mutant lines than their parents, possibly due to higher dependence on N_2 fixation than NO_3^- utilization.

6. NON-INVOLVEMENT OF ETHYLENE ON NITRATE INHIBITION OF SOYBEAN NODULATION

It has been reported in alfalfa that the inhibition of nodulation by nitrate was reduced by medication of ethylene production inhibitor aminoethoxyvinilglycine (Ligero et al. 1991). While the exogenous ethylene inhibited nodulation on the primary and lateral roots of pea (Lee and LaRue 1992ab). Ethylene is one of the important phytohormone regulating plant growth. Ethylene is produced through oxidative decomposition of 1-aminocyclopropane-1-carbosylic acid (ACC), and silver thiosulfate (STS) is a potent inhibitor of ethylene action in plant (Veen 1983). Sato et al. (1999c) investigated the effect of ethylene action on soybean nodulation using ACC and STS in relation to the inhibitory mechanism of nitrate using hypernodulation mutant NOD1-3 and the parent Williams. The hypernodulation mutant of soybean NOD1-3 and its parent Williams were cultivated in culture solution with or without NO_3^-, and ACC or STS were added in the solution. The nodule dry weight was decreased by both ACC and STS treatments, however, the ratio in nodule dry weight in total plant dry weight were not significantly influenced by these treatments with or without NO_3^-. Therefore, it was concluded that the decrease in nodule dry weight by ACC was caused by inferior growth. In soybean the depression of nodulation and N_2 fixation by nitrate is not mediated through ethylene action. Schmidt et al. (1999) also reported the independence of ethylene signaling on the regulation of soybean nodulation. Moreover, the nodulation of hypernodulation mutant was not specifically influenced by ACC treatments. This suggests that autoregulation may not be involved in ethylene action or transduction pathways in soybean plants.

Recently, defective long-distance auxin transport regulation was reported in the *Medicago truncatula* super numeric nodules mutant (Van Noorden et al., 2006). However, similar trend is not observed in hypernodulation mutants of soybean. Terakado et al (2005) reported that systemic effect of brassinosteroid on nodule formation in soybean after the foliar application of brassinolide and

brassinazaole, the inhibitor of brasinosteroid formation. In addition, they reported that shoot applied polyamines suppress nodule formation in soybean (Terakado et al, 2006). Suzuki et al reported that nodule number is controlled by the abscisic acid in *Trifolium repense* (white clover) and *Lotus japonicus* (Suzuki et al. 2004).

7. Effect of Salicylic Acid Supply on Nodule Formation of Hypernodulation Mutant

It is postulated that once the roots of soybean are infected with rhizobia, the infection signal molecule(s) is synthesized in the roots and transported to the shoot through xylem, and it induces the synthesis of autoregulation signal(s) which in turn transported to roots and depress further development of the nodule meristem. However, infection signal and autoregulation signals are not identified yet. Plants display a mechanism of resistance to pathogenic microbes. Salicylic acid is considered to be one of the endogenous signals in the systemic resistance to pathogen infection (Malamy et al. 1990, Metraux et al. 1990, Loak and Grant 2007). If some pathogenic microbes infect a plant, a hypersensitive reaction occurs in the infected portion of the plant where salicylic acid is synthesized. Then salicylic acid is transported to other plant part as a pathogen infection signal, and it induce gene expression of pathogen related proteins. On the other hand, jasmonic acid also induces the gene expression of pathogen related proteins as a signal of wound and viral infection (Gundlach et al 1992). Both the autoregulation of nodulation and the systemic acquired resistance to pathogenic microbes are mediated by some plant signals. Sato et al (2002) investigated the effect of salicylic acid supply on nodulation of soybean to determine whether salicylic acid affect the autoregulation of nodulation, using hypernodulation mutant lines, NOD1-3 and NOD2-4 and their parent Williams.

Seedlings of the hypernodulation mutant, NOD1-3 and NOD2-4 and the parent Williams were treated or not treated with a 100 μM salicylic acid solution at 5 days before the inoculation of *Bradyrhizobium japonicum* USDA110. The nodulation of Williams decreased dramatically by the addition of 100 μM salicylic acid. The nodule number with 100 μM salicylic acid was only 6, although it was about 40 in control plant without salicylic acid. The decrease in the nodule number was not caused by the reduction of the rhizobium number in the medium. Salicylic acid inhibited only early nodule formation and did not affect the growth of formed nodules. The inhibitory effect of salicylic acid on the nodulation of NOD1-3 and NO2-4 was significantly less pronounced than that in

Williams. These results indicate that salicylic acid may be involved in signal transmission in the autoregulation process.

8. INVOLVEMENT OF PHOTOASSIMILATE SUPPLY IN NODULE FORMATION IN HYPERNODULATION MUTANT OF SOYBEAN

Legume-rhizobia symbiosis depends on the exchanges of two major nutrients, nitrogen and carbon (photoassimilate). Therefore efficient supply of photoassimilate is very important to nodule initiation, nodule development, as well as maintenance of nitrogen fixation and transport of fixed nitrogen compounds. A continuous supply of photoassimilate is very important to support N_2 fixation, because shoot removal treatment quickly decreased the $^{15}N_2$ fixation both in Williams and NOD1-3 after 2hrs (Ohyama and Harper 1991).

Sato et al (1999) investigated the involvement of photosynthetic supply in changes of nodule characteristics of hypernodulation soybeans En6500 isolated from Japanese cultivar Enrei by sink-source manipulations. Because En6500 exhibit profound nodulation but smaller shoots than Enrei, they adjusted the sink and source size, either by decreasing the infection dose of *Bradyrhizobium japonicum* USD110 for En6500, or cutting the leaves of Enrei. To compare the nodule characteristics between the similar size of shoot with normal nodulation number, it was found that acetylene reduction activity per nodule dry weight, concentration and component ratio of leghemoglobin were similar. These results suggest that the specific characteristics of the nodules of hypernodulation mutant, such as low acetylene reduction activity per nodule dry weight, low leghemoglobin concentration and different component ratio of leghemoglobin and small infected region, is caused by the insufficient supply of photoassimilate to each nodule, due to excess nodule number. On the other hand, the partial tolerance of nodule growth and acetylene reduction activity per plant to nitrate was not directly involved in photoassimilate supply.

Initial nodule growth may be determined by photosynthetic product supply in relation to autoregulation of nodulation. Ito et al (2006a) investigated the allocation of photosynthetic products in soybean during the early stages of nodule formation. A time-course study was conducted in relation to nodule initiation. Whole shoots were exposed to $^{14}CO_2$ for 120 min and the distribution of radioactivity in each organ was determined. During the early stages of nodule formation at 4, 6, and 8 days after inoculation, the ^{14}C distribution to the

inoculated roots did not increase when compared with uninoculated control roots. In addition ^{14}C respired by underground parts was similar in both the inoculated and the control uninoculated roots. At 8 days after inoculation, the accumulation of starch and sugar was similar in both inoculated and uninoculated plants. These results indicated that photoassimilate allocation for nodular initiation does not increase markedly during the early stages of nodule formation. The rate of photoassimilate supply itself may not be a trigger to develop the arrested nodule meristems to functional nodules. After the emergence of nodules, photoassimilate allocation to the inoculated roots gradually increased. In addition, the consumption of current photoassimilate by the respiration of under-ground parts increased at day 12 after inoculation to support nodule growth before starting nitrogen fixation.

Ito et al. also reported the current photoassimilate allocation of hypernodulation mutant of soybean NOD1-3 in early stage of nodule formation (Ito et al. 2006b). Whole shoots were exposed to to $^{14}CO_2$ for 120 min and the distribution of radioactivity in each organ was determined. The ^{14}C distribution in the roots of 8 days after inoculation did not increase when compared with uninoculated control plants. In visualized images of radioactivity by imaging plate, nodules were observed as strong signal spots in underground organ. These results indicate that current photosynthate allocation to the inoculated root did not increase markedly during the early stages of nodule formation, while small nodules had already strong sink activity than the roots in 8 days after inoculation. This is faster than the parent Williams. To investigate carbon status of plants at 8 days after inoculation, the starch and sugar concentration in the plants were similar between inoculated and uninoculated roots of NOD1-3. It was concluded that increase in photoassimilate supply does not occur for nodule initiation in NOD1-3 until at 8 days after inoculation as same as the parent Williams.

9. CHARACTERISTICS OF EARLY GROWTH OF HYPERNODULATION MUTANTS

Characteristics of the initial growth of hypernodulation soybean lines, NOD1-3, NOD2-4, and NOD3-7 were compared with the parent Williams (Ito et al. 2006c) at 7 or 8 days after with or without inoculation of *Bradyrhizobium japonicum*. Total dry weight of each hypernodulation mutant lines were not significantly different form that of Williams in inoculated and uninoculated conditions. In inoculated conditions, nodule number at 8 days after inoculation

was higher in the order of NOD1-3 (56 nodules per plant), NOD2-4 (33), NOD3-7 (19) and Williams (17). In uninoculated conditions, the root growth of NOD1-3 and NOD3-7 were faster than that of Williams. Stem length and dry weight of NOD3-7 were lower than those of other lines, so shoot growth of NOD3-7 might be different from other lines. Seedling growth of NOD2-4 was very similar with that of Williams, except nodule number.

The characteristics of the initial growth of hypernodulation soybean lines, NOD1-3, NOD2-4, and NOD3-7 were compared with the parent Williams with or without 5 mM nitrate in the solution (Ito et al. 2007). When the plants were grown without inoculation, the total dry weight of all mutant lines was not different from Williams, both in the absence and presence of nitrate. These results indicate that the reduced accumulation of total dry matter of hypernodulation mutant lines may be secondary effect of excess nodule formation. When plants were grown with inoculation, the nodule number was decreased by the presence of nitrate in Williams, NOD1-3 and NOD2-4 but not in NOD3-7. NOD3-7 may be the most tolerant to nitrate inhibition of nodulation among the NOD mutant lines. In contrast, the leaf growth of NOD3-7 and NOD1-3 was different from the wild type. The expanded leaf was smaller, but the leaf emergence rate was faster compared with Williams under all the conditions. This suggests that NOD3-7 and NOD1-3 might decrease the ability for leaf expansion. A microscopic study showed that NOD1-3 and NOD3-7 lines produced small-size leaves due to the smaller number of leaf cells, compared with the Williams parent (Ito et al. 2008). This phenotype was not affected by inoculation with bradyrhizobiua or nitrate supply.

Recently, the genes were identified from hypernodulation lotus (*HAR1*) and soybean (*GmNARK*), which play important roles in the autoregulation of nodulation, and it was shown to encode a receptor-like kinase protein that contains a leucine-rich repeat (Krusell et al. 2002; Nishimura et al. 2002; Searle et al. 2003). These legume genes are homologous to Arabidopsis *CLAVATA1* (*CLV1*), which is involved in the control of cell proliferation in the shoot apical meristem (Clark et al. 1997). It is interesting that *HAR1/GmNARK* are expressed in most tissues except the shoot apical meristem (Nishimura et al. 2002; Searle et al. 2003), whereas *CLV1* is expressed in shoot apical meristem (Clark et al. 1997). The results of Ito et al. suggest that the protein coded by *GmNARK* may play some roles on leaf growth as well as nodule growth by autoregulation.

There is growing evidence that processes leading to nodule initiation and mycorrhizae are similarly regulated (Hirsch and Kapulnik 1998, Shrihari et al. 2000). Suppression of arbuscular mycorrhizal colonization and nodulation in split-root systems of alfalfa was observed (Catford et al. 2003), suggesting that

autoregulation mechanism may be shared in the rhizobial and the arbuscular mycorrhizal symbiosis. Recently, Meixner et al. (2007) reported that AMF precolonization to one part of split roots reduced secondary mycorrhization in wild type of soybean, while supernodulation mutant nts1007 from Bragg fail to control secondary mycorrhization. However, the supernodulating mutants En6500 from Enrei, NOD1-3 and NOD2-4 from Williams retained their ability to autoregulate arbuscular mycorrhizal fungi (AMF). These results showed that supernodulation mutants, despite a common nodulation phenotype, differ in their ability to autoregulate AMF colonization (Meixner et al. 2007).

10. PRACTICAL USE OF HYPERNODULATION SOYBEAN

The hypernodulation mutant lines of soybean may have some advantages, such as higher N_2 fixation or nitrate tolerant to nodulation. Wu and Harper (1991) evaluated the N_2 fixation potential and yield of hypernodulating soybean NOD1-3, NOD2-4 and NOD3-7 compared with the parent Williams. In the absence of N fertilizer, all hypernodulation mutants had greater N_2 fixation potential than did Williams in early growth stages. However, the seed yields from the hypernodulation mutants were 10 to 30% less than that from Williams. Suganuma et al. (2001) also compared the growth and N_2 fixation activity of NOD1-3 and Williams in sandy dune field. The relative dependence on N_2 fixation evaluated by simple relative ureide method was higher in NOD1-3 (65%) than that of Williams (58%), but total accumulation of N was lower in NOD1-3 compared with Williams due to inferior growth. The hypernodulation mutant lines have not been used for practical cultivar, but recently Sakukei No. 4 bred from En6500 has been used in agriculture. The Sakukei No. 4 is smaller than Enrei, however, by increasing planting density, the yield can be over than Enrei. Field assessment of supernodulating mutant of soybean showed that cropping is beneficial to subsequent cereal crops (Song et al., 1995), or mixed-cropping with sorghum (Ofosu-Budu et al. 1995).

Chapter 6

EFFECT OF NITROGEN NUTRITION ON SOYBEAN SEED STORAGE PROTEIN COMPOSITION

1. STORAGE PROTEIN IN SOYBEAN SEEDS

Soybean seed is one of the most important protein sources for human and live stocks. The storage proteins of soybean seeds mainly consists of glycinin and β-conglycinin. β-conglycinin comprises of three subunits, designated as α', α, and β subunits (Shuttuck-Eidens and Beachy 1985). Of the three subunits of β-conglycinin the β-subunit genes are known for their unique expression. Gayler and Sykes (1985) reported that soybean seeds cultured under sulphate deficiency showed a 40% decrease in the level of glycinin and a 3-fold increase in the amount of β-conglycinin. On the other hand, soybean cotyledons cultured in vitro in the presence of methionine lacked the β-subunit, and the gene expression was down regulated at the level of mRNA accumulation (Creason et al. 1983, Holowach et al. 1984, 1986). The β-subunit of β-conglycinin is especially low in sulfur amino acids, containing only one cysteine and no methionine residue in its mature form (Coates et al. 1985). Therefore, it may be reasonable to accumulate β-subunit of β-conglycinin in S-deficit conditions, and decrease it under S-suficient conditions.

2. DISCOVERY OF N REGULATION ON β-SUBUNIT OF β-CONGLYCININ

We happened to discover the lack of β-subunit of β-conglycinin in several non-nodulated soybean lines, although an electrophoretic protein band due to this protein was clearly detected in the corresponding nodulated isolines (Figure 61) (Ohtake et al, 1994). In two dimensional electrophoresis, several β-subunit polypeptide spots were present in nodulated line (T202) but corresponding spots were not detected in non-nodulated T201 seeds.

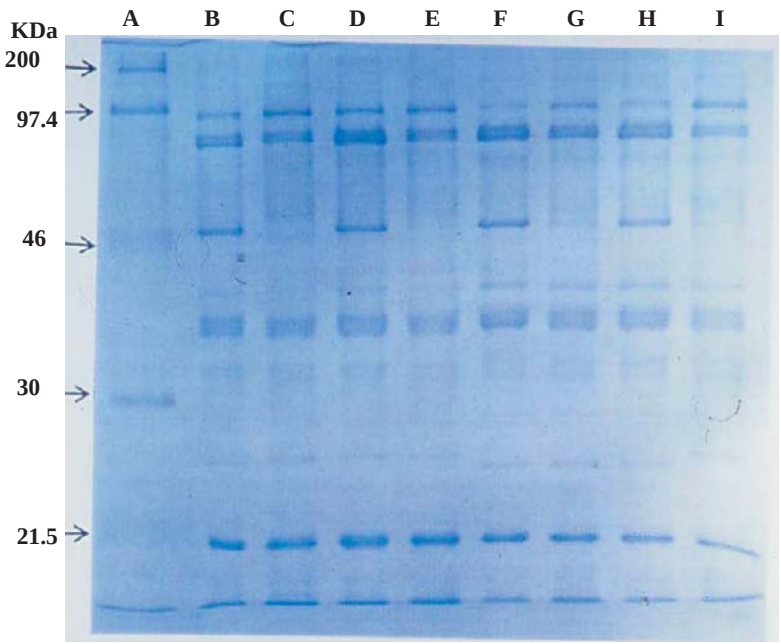


Figure 61. SDS-PAGE pattern of seed storage protein of nodulating and non-nodulating isolines. A: Molecular marker, B: T202, C: T201, D: Fujimijiro, E: Totan No. 90, F: Norin No. 2, G: Totan No. 89, H: A62-1, I A62-2. Nodulating lines (B,D, F, H), Non-nodulating lines (C,E,G,I) From Ohtake et al. 1994

Nodulated (T202) and non-nodulated (T201) isolines of soybean were cultivated in a rotated paddy field in Niigata, Japan (Ohtake et al. 1996). The pods and seeds were harvested at 7-days intervals until maturity, and the subunit compositions of seed storage proteins were analyzed by SDS-PAGE (Figure 62). The β-subunit of β-conglycinin could scarcely be detected in the non-nodulating

isoline, T201, at any period throughout seed development, although it was a major component in T202.

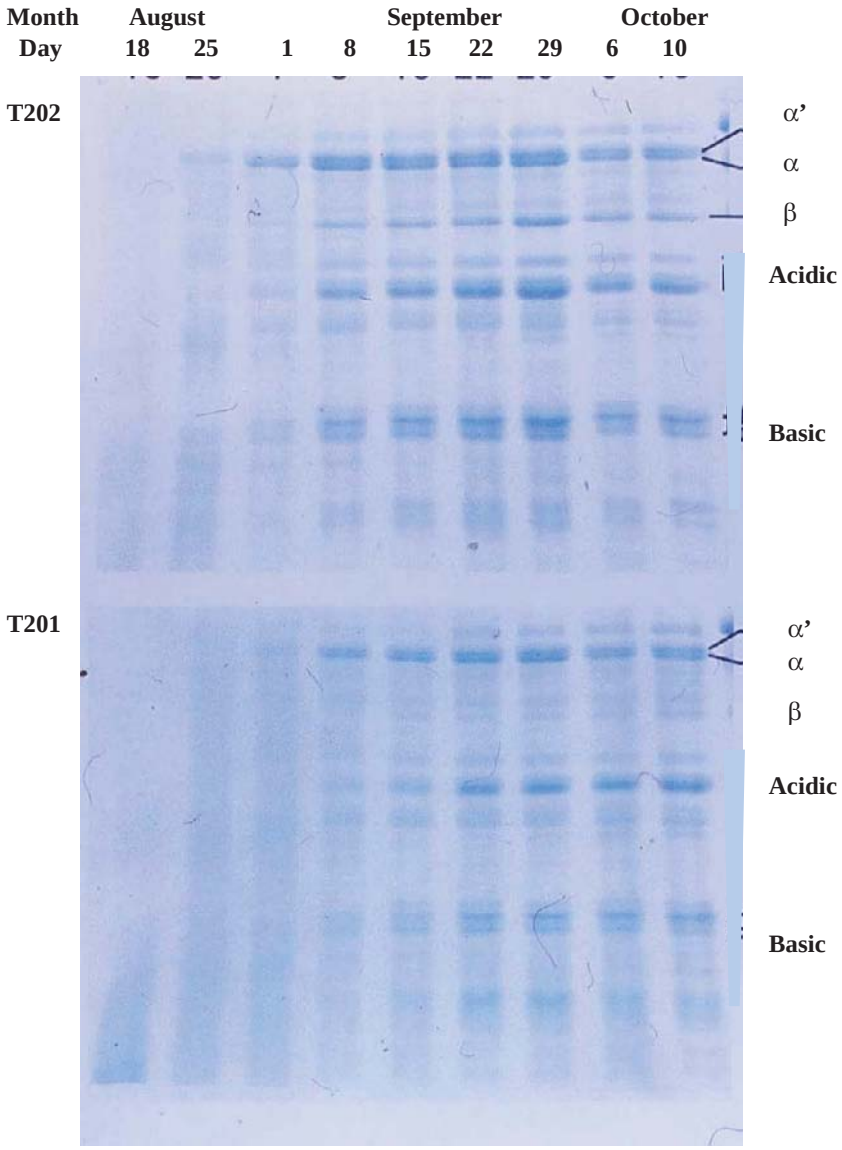


Figure 62. Changes in SDS-PAGE pattern of seed storage protein of nodulating (T202) and non-nodulating (T201) isolines. From Ohtake et al. 1997

Northern hybridization could not detect the β-subunit mRNA in immature T201 seeds, while it was pronounced in T202 (Figure 63). These results indicate that the suppression of the β-subunit in the non-nodulating isoline T201 is regulated at the level of mRNA accumulation. The α'- and α-subunits mRNAs were actively expressed in both lines. Total N concentration was consistently lower in non-nodulating T201 than nodulating T202, although no significant difference was observed in either the free amino acid or ureide concentrations in seeds.

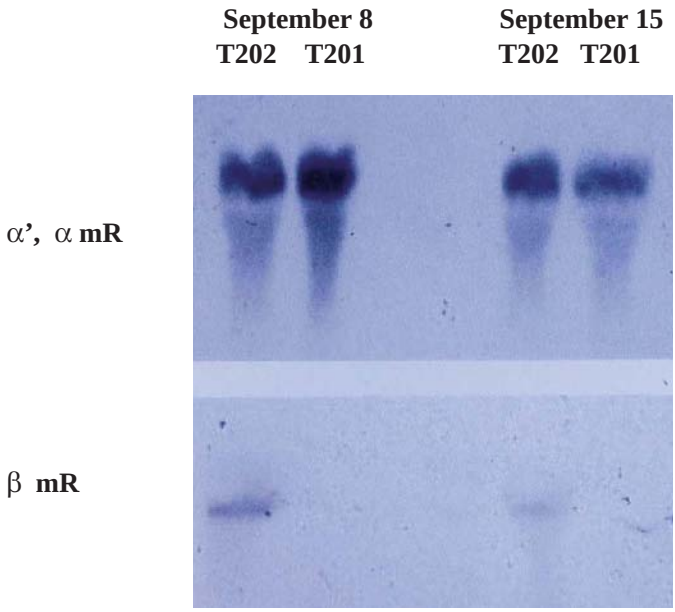


Figure 63. Accumulation of mRNA in the seed of nodulating (T202) and non-nodulating (T201) isolines. From Ohtake et al. 1997

The distribution of mineral elements and cell morphology in nodulated and non-nodulated soybean seeds were observed (Ohtake et al. 1997a). The distribution patterns of K, Ca, Mg, P, S, and N were similar between T201 and T202 seeds by EPMA. Protein bodies in cotyledon cells were observed by a microscope after a thin section was stained with coomassie brilliant blue solution (Figures 64, 65). The protein bodies of non-nodulated T201 were smaller than those of nodulated T202. Average cell size of T201 was significantly smaller about 30% than that of T202, while the total cell number in a seed was not significantly different between lines. This suggests that the nitrogen deficiency

did not affect cell proliferation of seeds, but it depressed the cell expansion and protein body accumulation.

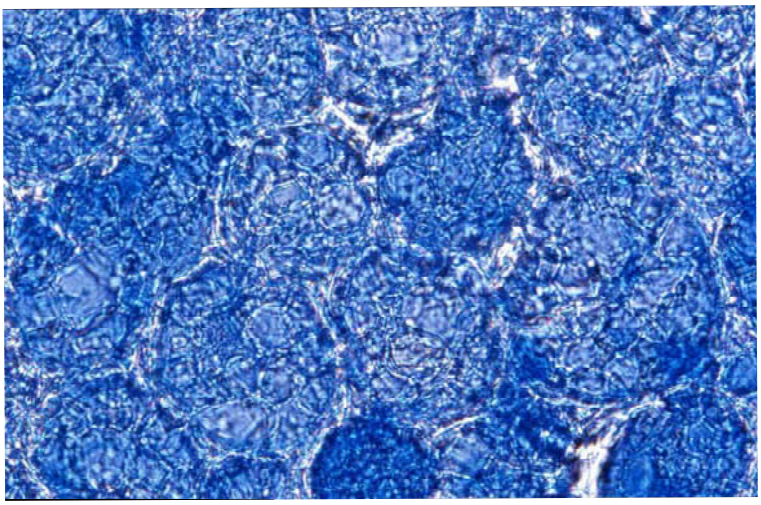


Figure 64. Microscopic observation of cotyledon cells of seed of nodulating T202. From Ohtake et al. 1997

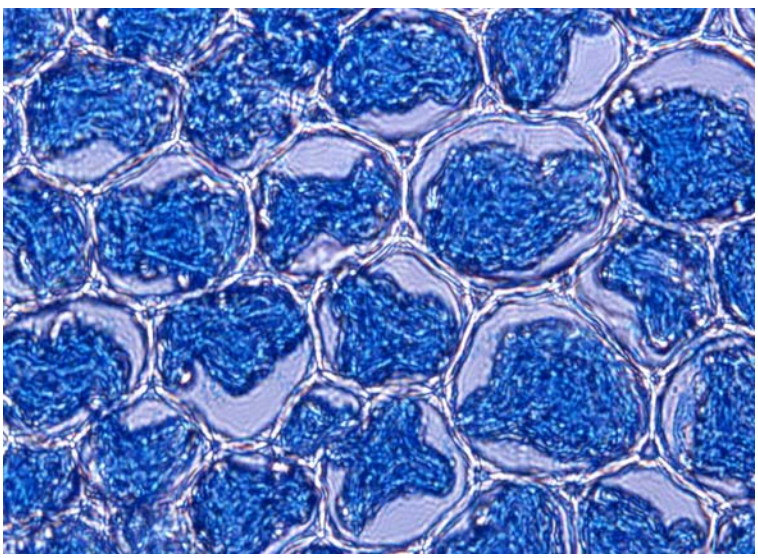


Figure 65. Microscopic observation of cotyledon cells of seed of non-nodulating T201. From Ohtake et al. 1997

Nitrogen regulation of storage protein subunit levels of soybean seeds was evaluated using T201 and T202 with solution culture in the greenhouse (Ohtake et al. 1997b). With a continuous 2 mM NO_3^- supply, seed dry weight and N concentration of the T201 were significantly lower than those in the T202 seeds, and the β-conglycinin proortion was lower in T201 than T202. When 5 mM NO_3^- was supplied, the subunit proportion of the seed storage protein was similar in nodulating and non-nodulating isolines. Furthermore, in T202 plants treated with 10 mM NO_3^- the proportion of β-conglycinin increased markedly.

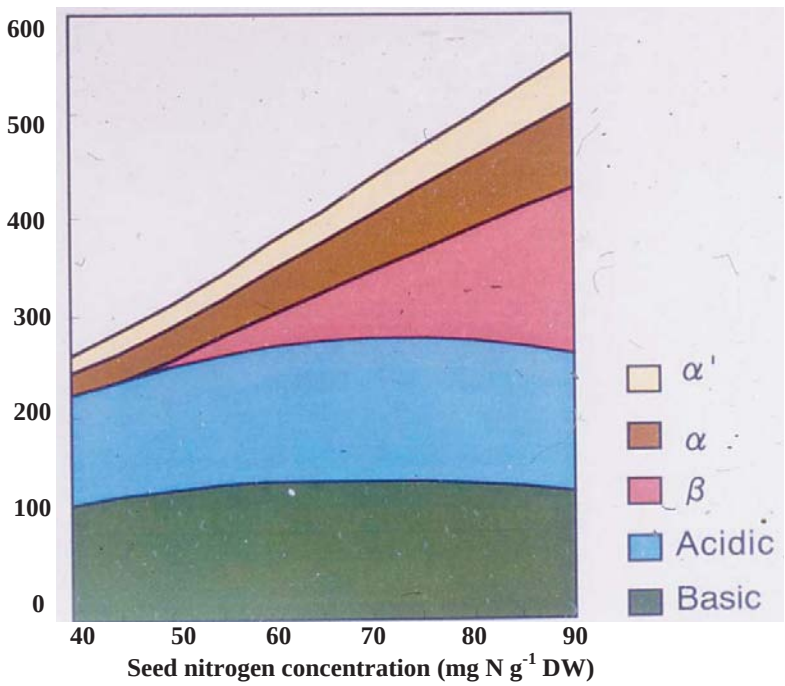


Figure 66. Relationship between seed storage protein accumulation and total nitrogen concentration in seeds. From Ohtake et al. 1997

The results indicate that non-nodulated T201 has a normal, non-defective, β-subunit genes, and that limited N availability decreases accumulation of β-conglycinin, whereas high N availability decreases accumulation of β-conglycinin in soybean seeds, irrespective of whether N was derived from N_2 fixation or from NO_3^- absorption. Figure 66 shows the relationship between seed storage protein composition and the total N concentration of seeds, which were harvested from soybean plant with various N conditions. The concentration of glycinin was

relatively constant irrespective of total N concentration of seeds. On the other hand, the accumulation of β-conglycinin especially the β-subunit was significantly influenced by N concentration, and it was lost by severe N deficiency and it was stored with excess N concentrations. This was the fist discovery of N regulation of soybean storage protein accumulation. This result was confirmed by following researches (Paek et al. 1997, Nakasathien S, 2000).

Ohtake et al. (2002) reported the effect of short-term application of nitrogen on the accumulation of β-subunit of β-conglycinin mRNA and protein in intact seeds and *in vitro* cotyledon culture system. Non-nodulating isoline (T201) were cultivated with low N (0.5 mM NO_3^-) conditions, and they were transferred to N-sufficient condition (5 mM NO_3^-) conditions. The β-subunit mRNA and the protein were detected in immature seeds within 2 days after transfer to 5 mM NO_3^- medium. Among the free amino acids in immature seeds, asparagine concentration increased rapidly within 2 days. In the in vitro cotyledon culture system, the application of glutamine induced the accumulation of β-subunit mRNA within 12 hr, while asparagine did not induce the accumulation of β-subunit mRNA for 7days. From these results, it was concluded that the accumulation of β-subunit mRNA and the protein may be regulated by glutamine concentration, or its metabolites or related compounds. Rainbird et al. (1984) reported that glutamine was the principal N to cotyledon, contributing 55% of the embryo nitrogen requirement, and 20% from asparagine, and negligible from ureides, allantoin and allantoic acid. Haga and Sodek (1987) also reported that glutamine was the most efficient source in terms of protein accumulation in the cultured soybean cotyledons, while asparagine was less efficient and allantoin was a poor source of nitrogen.

Concerning to C supply to the seeds, sucrose is a principal C source for pods and seeds (Fellows et al. 1978). Yanagisawa et al. (1986) showed by $^{13}CO_2$, $^{15}N_2$ and $^{15}NO_3^-$ labeling experiment that the assimilated ^{13}C was rapidly transported to each organ compared with ^{15}N from $^{15}N_2$ fixation and $^{15}NO_3^-$ absorption. Yamagata et al. (1987) reported that 96% of C in matured soybean seed carbon derived from photosynthate after anthesis.

Ohtake et al. (2001b) reported that the rapid N transport to pods and seeds in N-deficient soybean plants compared with N-sufficient plant. Non-nodulated soybean plant (T201) was cultivated hydroponically under N-sufficient or N-deficient conditions. ^{13}N or ^{15}N labeled NO_3^- was supplied from cut end of the stems, and the accumulation of nitrate derived N was compared.

Figure 67 shows the outline of the N flow in soybean seed (cotyledon). Ureide is transported from the root nodules via xylem and accumulated in the pod.

Allantoin and allantoic acid are metabolized to amino acids and excreted into seed coat. Asparagine from roots via xylem or from leaves via phloem is also metabolized to amino acids then provided into the apoplast space between seed coat and cotyledon. The cotyledon cells absorb amino acids and they synthesize storage proteins and accumulate them into protein bodies.

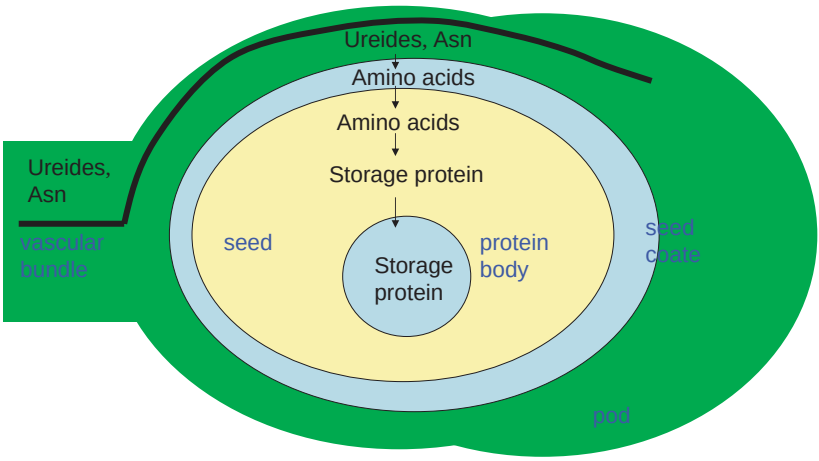


Figure 67. A model of N flow in soybean seed.

Chapter 7

DEVELOPMENT OF NEW FERTILIZATION TECHNIQUE TO PROMOTE NITROGEN FIXATION AND SEED YIELD

1. NITROGEN FERTILIZATION IN SOYBEAN CULTIVATION

Soybean requires a large amount of N relative to other crops. About 80 % of total N was assimilated after initial flowering stage (R1). Sole N_2 fixation is generally insufficient to support vigorous growth of shoot and roots, which results in the reduction of plant growth and seed yield. On the other hand, a heavy supply of N fertilizer often depresses nodule development and N_2 fixation activity and induces nodule senescence, which also results in the no-effect or sometimes in reduction of seed yield. Therefore, nitrogen fertilizer is not applied for soybean cultivation or only a small amount of N fertilizer is applied as a "starter N" to promote initial growth. Basal dressing of ammonium sulfate (about 10-30kgN ha^{-1} of ammonium sulfate) is applied in Japan.

Top dressing of N fertilizer sometimes gave positive effect on seed yield, but not consistent. Gan et al (2003) reported that N top dressing of urea (50 kgN ha^{-1}) at either V2 or R1 stage significantly increased N accumulation, yield and total amount of N_2 fixed, although the N top dressing at either R3 or R5 stage did not show this positive results in China. Takahashi et al (2006) reported the effect of basal side-dressing of various types of coated urea fertilizer on shoot growth and yield of soybean. The yield was significantly higher in all the coated urea basal

side-dressing compared with control, particularly in the CUS120 which release urea sigmoidally from 60 to 120 days after planting.

We have developed a new fertilization technique for soybean cultivation to supplement N during seed filling stage without concomitant depression of N_2 fixation by deep placement (20 cm depth from soil surface) of slow release N fertilizers, coated urea and lime nitrogen, including calcium cyanamide as a major component. We analyzed the beneficial effects from both plant nutrition and soil analysis sides. We would like to introduce the outline of our study and review the characteristics of soybean N nutrition and N fertilization to promote high and stable soybean seed production.

2. DEEP PLACEMENT OF COATED UREA

A polymer coated controlled release N fertilizer (commercial name LP in Japan or MEISTER outside Japan) has been invented by Fujita and coworkers (Fujita and Shoji 1999) Linear types of coated urea were first marketed in 1982. This type of fertilizer has spherical shape about 3mm diameter with 50-60 μm coat thickness which consists of polyolefin (polyethylene), ethylene vinyl acetate and talc mineral. An example is shown in Figure 68.

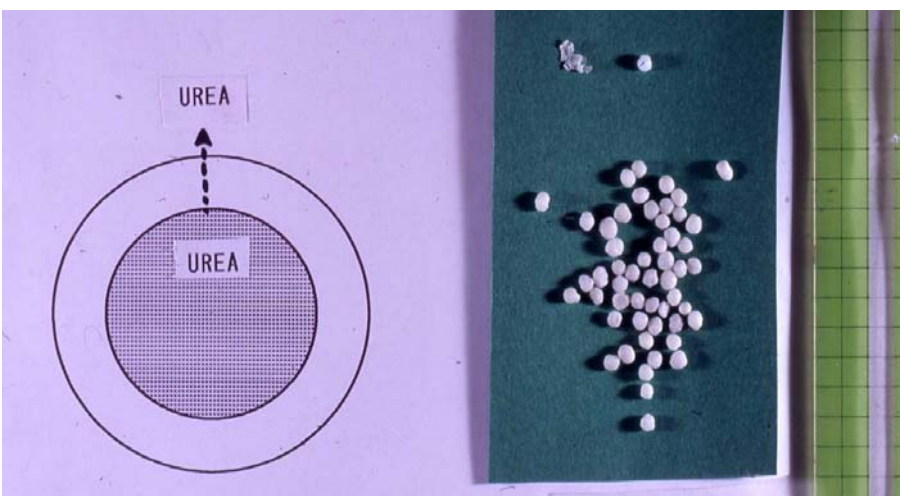


Figure 68. Coated urea fertilizer.

Different from chemically synthesized slow release N fertilizer such as CDU (Crotonylidene diurea), IBDU (Isobutylidene diurea), the N release rate from coated urea is temperature dependent and not affected by other chemical, physical and biological conditions, and the release pattern can be predicted as a function of temperature and time period after application. Since the release of N from the fertilizer meets the plant N demand, and the fertilizer efficiency (recovery rate of N in plants from fertilizer) is high, the use of coated urea can reduce the environmental problems by decreasing nitrate accumulation and leaching in the soil. Also the use of coated urea saves the labor of farmers by eliminating top dressing to supply N during later stage.

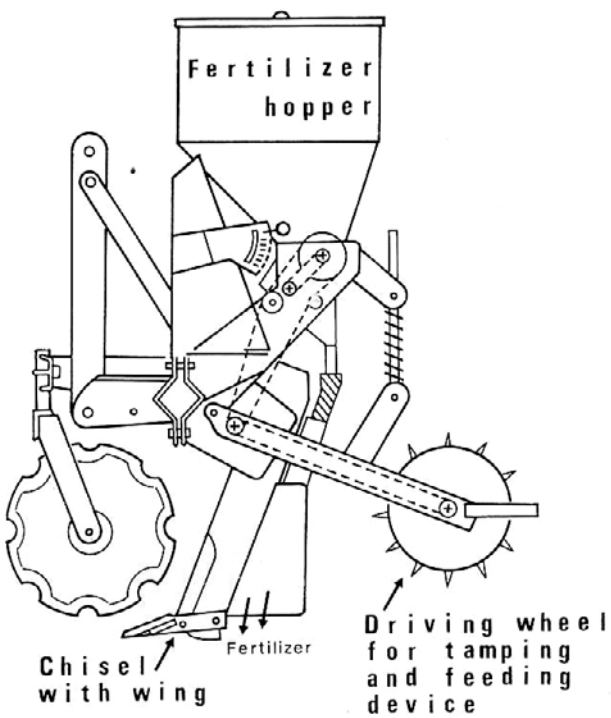


Figure 69. Fertilizer injecter for deep placement. From Takahashi et al. 1993

Takahashi *et al.* (1991a, 1992, 1993b, 1994) developed a new fertilization technique for soybean to supplement N during the seed filling stage without concomitant depression of symbiotic N_2 fixation by deep placement of coated

urea slow release N fertilizer. They applied 100 kg N ha^{-1} coated urea by deep placement (20 cm depth from soil surface) using fertilizer injector devised by Shioya (Figure 69).

They used CU-100, a 100 day type coated urea, the commercial name "LP-100" produced by Chisso Co, Japan. CU-100 linearly releases urea and 80 % of which is released in 100 days in water at 25 ^{o}C (Figure 70).

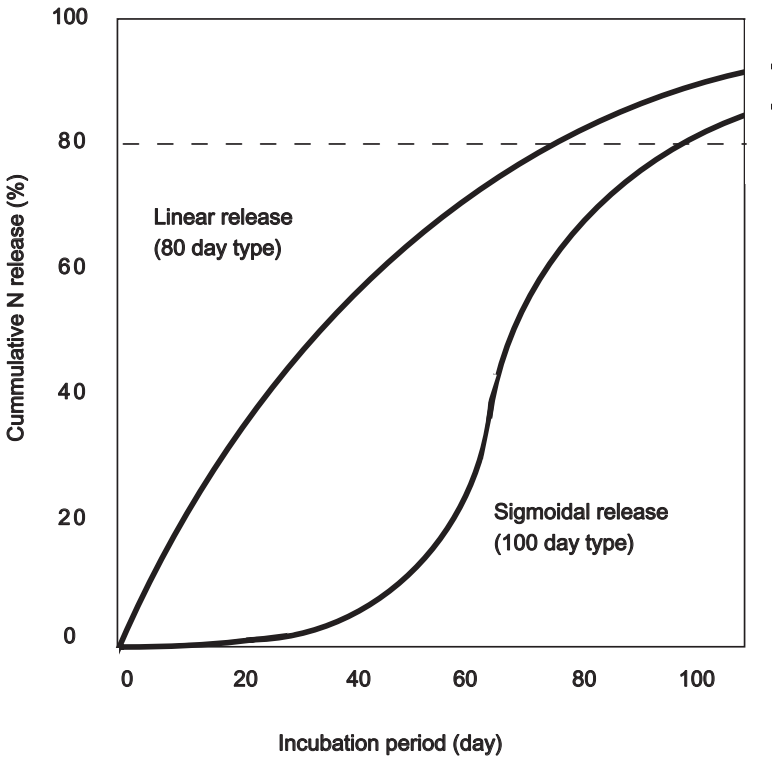


Figure 70. N release patterns of linear type and sigmoidal type ofcoated urea . From Ohyama et al. 2004

Fertilizer experiments were carried out from years 1989 to 1991 in the field, which had been converted from a paddy rice field in the previous year. The soil is a Fine-Textured Gray Lowland soil. Average chemical properties of the soil were as follows: texture; CL, pH(H_2O); 6.6, CEC; 28.8 (cmol(+) kg^{-1}), total carbon content; 10.9 g kg^{-1}, total N content; 1.02 g kg^{-1}, amount of mineralized N determined by the incubation of air dry soil under upland conditions for 4 weeks at 30 ^{o}C; 47 mg kg^{-1} (Takahashi et al. 1994).

Conventional basal dressing of ammonium sulfate (16 kgN ha^{-1}), fused magnesium phosphate (60 kgP_2O_5 ha^{-1}) and potassium chloride (80 kgK_2O ha^{-1}) fertilizers were mixed in plow layer (0-13 cm depth) of all experimental plots. Three fertilizer treatments were conducted as follows. Control; no additional fertilizer. Deep placement of 100 day type coated urea; basal deep placement (20cm depth from soil surface) of CU-100 below sowing planting line (100 kgN ha^{-1}). Top dressing of 70 day type coated urea; top dressing of CU-70 (100 kgN ha^{-1}) just before the flowering stage with intertillage and earthing up. The seeds of soybean (*Glycine max* [L.] merr. cv. Enrei) were sown by single stem training (75cm x 15cm; planting density, 8.9 plants m^{-2}).

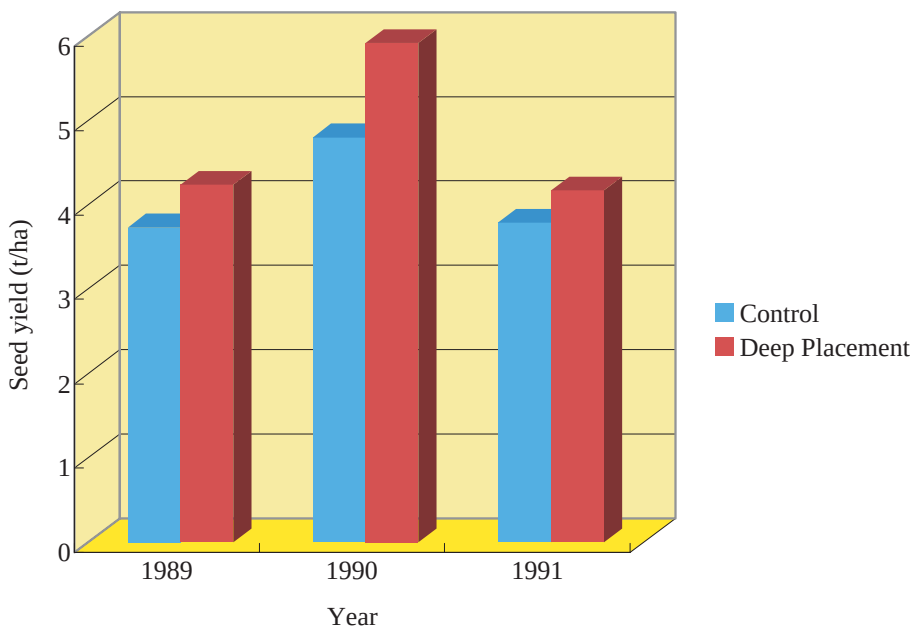


Figure 71. Seed yield of soybean plants cultivated in rotated paddy field with or without (control) deep placement of coated urea (LP-100) for three years. From Takahashi et al. 1995

As shown in Figure 71 the seed yield was significantly higher in the plants with the deep placement of CU-100 than control in each year. The seed yield was from 10 (1991) to 23 % (1990) higher in deep placement than the control treatments. The promotion of leaf growth and retardation of leaf senescence were observed during the maturing stage. In 1990, the seed yield was very high about 6 t ha^{-1} in deep placement due to the favorable climatic conditions compared with

years 1989 and 1991. The absorption efficiency of fertilizer N determined by ^{15}N labeled fertilizers was calculated from recovery of ^{15}N in the shoots at R7 stage (Figure 72). In 1990, the absorption efficiency at R7 from the deep placement of CU-100 was 62 %, which was much higher than the top dressing of CU-70 (33 %) and basal application of ammonium sulfate (9 %).

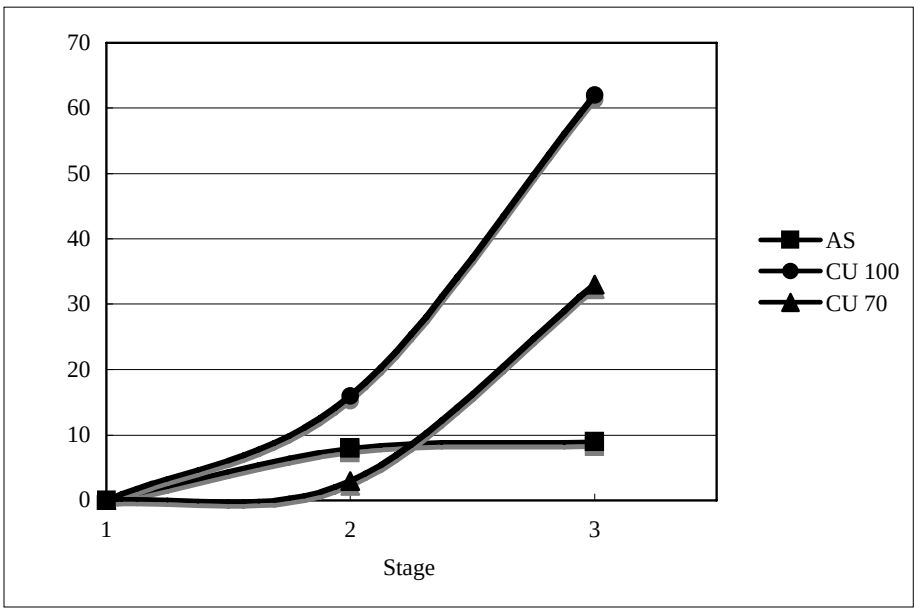


Figure 72. Recovery rate (%) of fertilizer N from basal deep placement of CU100, top dressing of CU70 and basal dressing of ammonium sulfate.Stage 1; Planting, Stage 2; R3, Stage 3; R7 From Takahashi et al. 1995

In the experiments conducted by Takahashi *et al.* (1991a,1992,1994, 1995) included the top dressing of linear type coated urea (CU-70, 100 kgN ha^{-1}) just before flowering stage with intertillage and earthing up. Although CU-70 is also control released N fertilizer, the top dressing of CU-70 did not consistently increase seed yields, due to depression of N_2 fixation activity during reproductive stage from the analysis. Fertilizer efficiency was about 30 % and much lower than deep placement of CU-100.

3. N ORIGIN OF PLANTS BY DEEP PLACEMENT OF COATED UREA

By periodical sampling of soybean plants and xylem sap, quantitative estimation of the seasonal changes in N_2 fixation activity and N absorption rate has been published (Takahashi et al 1992). Table 1 shows the estimation of daily N_2 fixation activity and N absorption rate of soybean plant in 1990 by simple relative ureide method (Takahashi et al. 1993a, FNCA 2006). Compared with control plants, the plants with deep placement of CU100 show higher N accumulation rate at any stages. It is remarkable that the deep placement of coated urea did not depress N_2 fixation activity. In addition N absorption rate was always higher in deep placement than that of control plant at any stages.

Table 1. Estimation of daily N_2 fixation activity and N absorption rate of soybean plants estimated by simple relative ureide method.

Period (DAS)	N accumulation (mg m^{-2} day^{-1})		N Fixation (mg m^{-2} day^{-1})		N Absorption (mg m^{-2} day^{-1})	
	Cont	DP	Cont	DP	Cont	DP
0-34	55	70	36	41	19	29
34-44 (R1)	110	251	83	172	27	79
44-60 (R3)	352	361	310	274	42	87
60-74 (R5)	478	591	414	436	64	154
74-116(R7)	423	485	279	276	143	210

DAS; days after sowing, Cont; control treatment, DP; Deep placement of coated urea. (Recalculated from the data in Takahashi *et al.* (1992))

Table 2 shows the comparison of the total amount of fixed N and absorbed N from fertilizer N and soil N at R7 stage. The plants with deep placement of CU100 absorbed about 6.4 gN m^{-2} from fertilizer and it was much higher than that (0.15 gN m^{-2}) in the control plants. The fertilizer efficiency of deep placement of CU100 was very high about 64 %, although that from basal application of ammonium sulfate was less than 10 %. It was remarkable to note that the amount of fixed N in deep placement of CU100 was about 25.2 g m^{-2} and not lower than that in control plants (24.5 g m^{-2}). This indicates that the deep placement of CU100 did not depress N_2 fixation activity with efficient supplement of N from the lower part of the roots. Consequently, the plant growth and seed yield were much exceeded mainly by increasing pod number in comparison to control cultivation without deep placement. Board and Tan (1995) reported that source strength influenced pod umber from R1 to 10 to 12 days after R5 stage. Pod per

reproductive node were regulated by pod initiation (pods at least 0.5 cm long) and / or abortion of initiated pods.

Table 2. Comparison of the amount of fixed N, absorbed fertilizer N and soil N and total accumulated N between control and deep placement of coated urea at R7 stage (116DAS).

Treatment	Total N ($g\ m^{-2}$)	Fixed N ($g\ m^{-2}$)	Fertilizer N ($g\ m^{-2}$)	Soil N ($g\ m^{-2}$)
Control	33.04	24.52	0.15	8.37
Deep placement	39.34	25.21	6.39	7.74

Recalculated from the data in Takahashi *et al.* (1992)

It was observed that the CU100 deep placement increased root growth and water and nutrient absorption activity revealed by rubidium uptake tracer experiment (Takahashi et al. 1991b). Owing to the promotion of root growth and absorption activity in the deep place with supplementing N fertilizer without depression of N_2 fixation, plant growth was promoted from early vegetative stage till late maturing stage. Leaf area index (LAI) and chlorophyll content were always higher in CU100 deep placement than those in control, and the leaf senescence was retarded at R7 stage (Takahashi et al. 1994) It was suggested that leaf senescence and N redistribution during seed filling may limit soybean yield by restricting the seed filling period (Hayati et al. 1995).

The seed quality related to the chemical composition of soybean cultivated with deep placement of coated urea was compared with control seeds (Ohyama et al. 1994b). The N concentration in the seeds was almost the same as in the case of control seeds. In addition, the concentration of oil, starch, and minerals (P, K, Ca, Mg, Na and Fe) in both seeds were almost the same between treatments.

3. FATE OF N FROM COATED UREA IN SOIL

Takahashi *et al.* (1993b) analyzed the concentration of urea, ammonium and nitrate in the upper 0-10cm and lower 15-25cm layers of control and deep placement of CU100 treatments. In the upper layer, the concentration of urea and nitrate was very low (less than 10 $mgN\ kg^{-1}$ soil) both in control and deep placement of CU100 treatments. However, the accumulation of ammonium (up to 150 $mgN\ kg^{-1}$) and nitrate (up to 50 $mgN\ kg^{-1}$) was observed in August in the lower layer of deep placement of CU100. Although the urea released from coated urea was rapidly hydrolyzed to ammonia, NH_4^+-N could not be easily nitrified in

the deep soil layers of the converted rice field owing to the low activity of nitrification and restricted O_2 supply. Also both ammonium and nitrate concentrations in the surface layer did not change by deep placement of CU-100 treatment. As a result, the nodulation and N_2 fixation in the surface layer were not depressed, and rather promoted through the improvement of the plant growth and photosynthetic activity.

4. THE MECHANISM OF PROMOTION OF DEEP PLACEMENT OF COATED UREA FOR SOYBEAN GROWTH AND SEED YIELD

The mechanism of promotion of deep placement of coated urea for soybean growth and seed yield is summarized in Figure 73.

a. Deep placement of coated urea slowly releases urea inside and the urea is rapidly hydrolyzed to ammonium and the ammonium does not easily leach out from the fertilization sites.
b. The abundant supply of N in the lower layer promotes the root growth, and water and nutrients absorption activity and fertilized N is efficiently absorbed from the lower roots.
c. The abundant supply of N from lower part of roots promotes leaf growth and extends the photosynthetic activity until maturing stage. The leaf area and chlorophyll content was higher in the leaves of deep placement than those in control ones.
d. Abundant supply of photoassimilate to nodules supports the nodule growth and N_2 fixation activity for an extended period during maturing stage.
e. Continuous supply of N from nodules and roots with increased photoassimilate supply promotes seed yield without decreasing the quality.

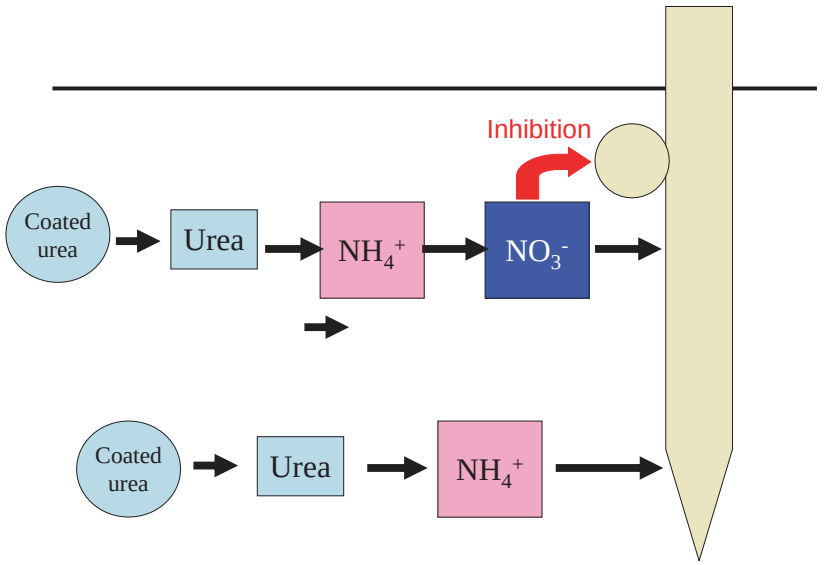


Figure 73. Effect of deep placement of coated urea.

5. Deep Placement of Lime Nitrogen with Different Inoculation Methods

Recently, Tewari *et al.* (2002, 2003, 2004a) investigated the effects of deep placement of lime nitrogen (calcium cyanamide, $CaCN_2$,) in comparison with coated urea. The fertilizer experiments were combined with new inoculation method of bradyrhizobia using a paper pot inoculation method. All the experiment was carried out in 2001 in three different sites in Niigata Prefecture. A first cropping field after reclamation with the dressing of mountain soil without indigenous bradyrhizobia (Tewari et al. 2002), a rotated paddy field in Niigata Agricultural Research Institute (Tewari et al. 2003), and a sandy dune field of Faculty of Agriculture in Niigata University (Tewari et al. 2004a). The effects of the application of different fertilizers, urea, coated urea and lime nitrogen on the growth, N accumulation and N_2 fixation activity of soybean plants were compared.

Lime nitrogen contains about 60% of calcium cyanamide ($CaCN_2$) with calcium oxide and carbon, and the N content is about 20-23%. It corresponds chemically and physiologically to a basic fertilizer and it neutralizes the soil acidity. After application to soil, the calcium cyanamide is converted to urea, which is again degraded into NH_3 and CO_2. Dicyandiamide contained in lime

nitrogen or formed during the degradation of calcium cyanamide retards the oxidation of NH_4^+ to NO_3^-, since it is a potent nitrification inhibitor. Therefore the ammonium produced by $CaCN_2$ decomposition persists for longer period of time and the nitrate concentration remains low in the soil. It is expected that the inhibition of nodulation and of the N_2 fixation activity may be alleviated by low level of nitrate accumulation in the soil. Also this fertilizer exerts some hormonal effects on plants and is used for controlling soil diseases caused by bacteria and fungi.

Nitrogen (ammonium), phosphorus, potassium and calcium fertilizers in basal application (16 kgN ha^{-1}, 60 kgP_2O_5 ha^{-1}, 80 kgK_2O ha^{-1}, 1000 kg $Ca(OH)_2$ ha^{-1}, respectively) were incorporated into the plow layer at about 0-10 cm depths for all the experimental plots. Thereafter, deep placement was performed at a 20 cm depth under the planting spot with different treatments as follows. Control; without additional fertilizers, Urea; Deep placement of urea 100 kgN ha^{-1}, CU-100; Deep placement of 100-day type coated urea, 100 kgN ha^{-1}, $CaCN_2$; Deep placement of lime nitrogen, 100 kgN ha^{-1}.

In each of these fertilizer treatments, IPP (Inoculated paper pot), DT (Direct transplantation of inoculated seedlings without paper pot) and NIPP (Non-inoculated paper pot) seedlings were transplanted in separate plots. Paper pots (height 13.5 cm, diameter 3 cm) are made of a biodegradable paper designed to break down in the field. The pots are open at the bottom to allow root expansion below the pot, although radial expansion of the soybean root system through the paper was restricted in some fields. Inoculation of legume seed is most conventional way of introducing effective rhizobia to soil, but full potential of incoluation is not always achieved (Deaker et al. 2004). Senoo et al (2002) reported that soil aggregate-based inoculant is more effective for soybean and red kidney bean growth and nodule occupancy.

As shown in Figure 74, paper pot was filled with vermiculite and a seed was planted in each pot, and followed by inoculation of one ml of suspension of *Bradyrhizobium japonicum* USDA110 about 10^8 cells ml^{-1}. Since the bradyrhizobium population increases about 100 times in vermiculite for a few weeks (Minagawa et al. 1997), efficient infection of inoculated bradyrhizobia can also be expected by paper pot inoculation with vermiculite. The use of paper pots leads to uniform germination and seedling growth, and transplantation protects the seeds from feeding by pigeons. Therefore, injury and delay in germination, which often result in severe damage to soybean cultivation, can be avoided. In these experiments holes at a depth of about 20 cm were dug with a scoop at the transplanting sites. Thereafter, the previously weighed fertilizers urea, CU-100

and $CaCN_2$ were applied in the respective holes just under the seedling placement. Then the seedlings were transplanted above the fertilizer application sites.

Figure 74. Paper pot inoculation method.

Table 3 summarized the seed yield of three different sites in 2001. In the reclaimed field, the most conspicuous effect was observed by inoculation and deep placement (Figure 75). The seed yield was higher in rotated paddy field where mineralized N from soil organic matter is relatively abundant compared with reclaimed field and sandy dune field where soil fertility is very low. Concerning to the inoculation method, IPP (inoculated paper pot) tended to show the highest seed yield than DT and NIPP treatments. Especially in newly reclaimed field with dressing of mountain soil without indigenous rhizobia, the inoculation by IPP or DT promoted seed yield more than twice as much in the control treatment. Among the inoculation methods, the IPP and DT seedlings

showed a higher seed yield than the NIPP seedlings; especially the difference was statistically significant in the CU-100 and $CaCN_2$ treatments to control plants.

Table 3. Seed yield of soybean with fertilizers and inoculation treatments at different fields in 2001

Inoculation Methods	Fertilization Deep Placement	Seed yield (t ha^{-1}) in Experimental fields		
		Rotated paddy field	Reclaimed field	Sandy dune field
NIPP	Control	2.88 b	0.78 b	1.72 b
	Urea	4.53 a	2.86 a	2.46 a
	CU-100	4.29 a	3.58 a	2.49 a
	$CaCN_2$	4.60 a	3.40 a	2.50 a
DT	Control	3.14 b	1.94 b	1.91 b
	Urea	4.22 ab	3.36 a	2.62 a
	CU-100	5.35 a	3.97 a	2.71 a
	$CaCN_2$	5.41 a	3.56 a	2.67 a
IPP	Control	3.31 b	2.01 c	1.83 b
	Urea	4.67 b	2.90 b	2.73 a
	CU-100	6.04 a	4.00 a	3.05 a
	$CaCN_2$	6.12 a	4.19 a	3.32 a

NIPP; non-inoculated paper pot, DT; direct transplanting of inoculated seedlings, IPP; inoculated paper pot, Means followed by the same letter are not significantly different by 5% level in the same inoculation method in the same field.
(From Tewari *et al.* 2002, 2003, 2004)

As a consequence of the promotion of N acquisition and plant growth, significantly higher seed yields in the rotated paddy field were obtained with the deep placement of $CaCN_2$ IPP (6.12 t ha^{-2}) and CU-100 IPP (6.04 t ha^{-2}), compared with the Urea IPP (4.67 t ha^{-2}) and Control IPP (3.31 t ha^{-2}) treatments (Figure 76), (Tewari et al 2003). The promotive effects of slow release fertilizers on soybean seed yield resulted in the yield about 49-85 % higher than that of the control yield. The similar effect was observed in reclaimed field and sandy dune field, where deep placement of lime nitrogen was almost the same or better seed yields.

The promotive effect on seed yield (dry weight) of the deep placement can be analyzed based on the yield components in paddy field (Tewari et al. 2004a), The number of pods per m^2 with the slow release fertilizers CU-100 and $CaCN_2$ was significantly higher than that in the Control and Urea treatments. The higher yield by deep placement of CU-100 and $CaCN_2$ was mainly due to the significant increase in the total pod and seed numbers. The seed weight and N content were

also found to be highest with the $CaCN_2$ treatment followed by the CU-100 treatment.

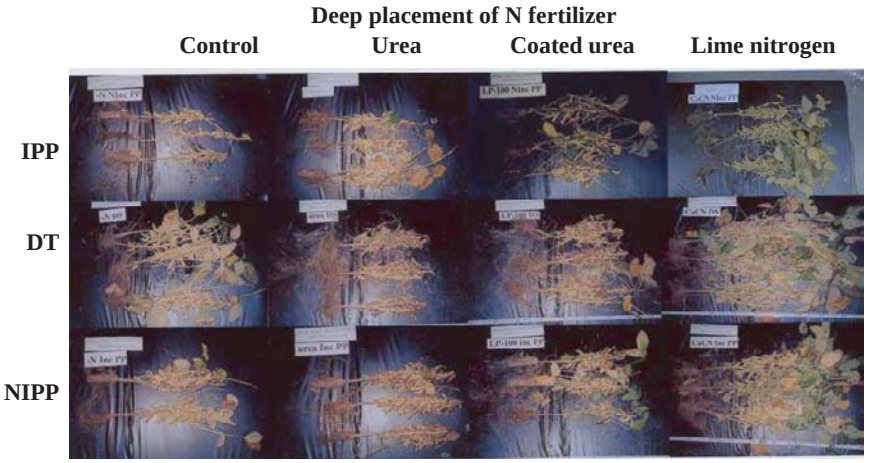


Figure 75. Effect of deep placement of urea, coated urea or lime nitrogen and paper pot inoculation method in Yamakita. IPP: Inoculation paper pot transplantation, DT: Direct transplanting with seed inculation, NIPP: Non-inoculated paper pot transplantation. From Tewari et al. 2002

The N content of the shoot vegetative organs was significantly higher in the deep placement of N fertilizers compared with the control, both at the R1 and at R7 stages. At the R1 stage, it was highest in the Urea with DT treatment but later at the R7 stage, the N content increased with the application of slow release fertilizers, $CaCN_2$ and CU-100. This may be due that urea is a readily available N fertilizer and CU-100 and $CaCN_2$ are slow release N fertilizers. The total N content of the shoot organs including leaves, stems, pods, and seeds was also found to be highest with $CaCN_2$ among the fertilizer treatments and with the IPP among the inoculation methods.

The %Ndfa estimated by the simple relative ureide method in the Control accounted for 50, 54, and 54 % in the IPP, DT and NIPP methods, respectively. On the contrary, in the plants with deep placement of CU-100 and $CaCN_2$, the %Ndfa ranged between 60-79 % in all the inoculation methods, suggesting that deep placement of these slow release fertilizers promoted the N_2 fixation activity. In the deep placement of Urea, the %Ndfa was found to be as low as 47 and 43 % in the IPP and NIPP methods, respectively, suggesting that N_2 fixation activity was inhibited by this treatment. When the inoculation methods were compared, the DT plants showed a higher %Ndfa than the IPP and NIPP plants.

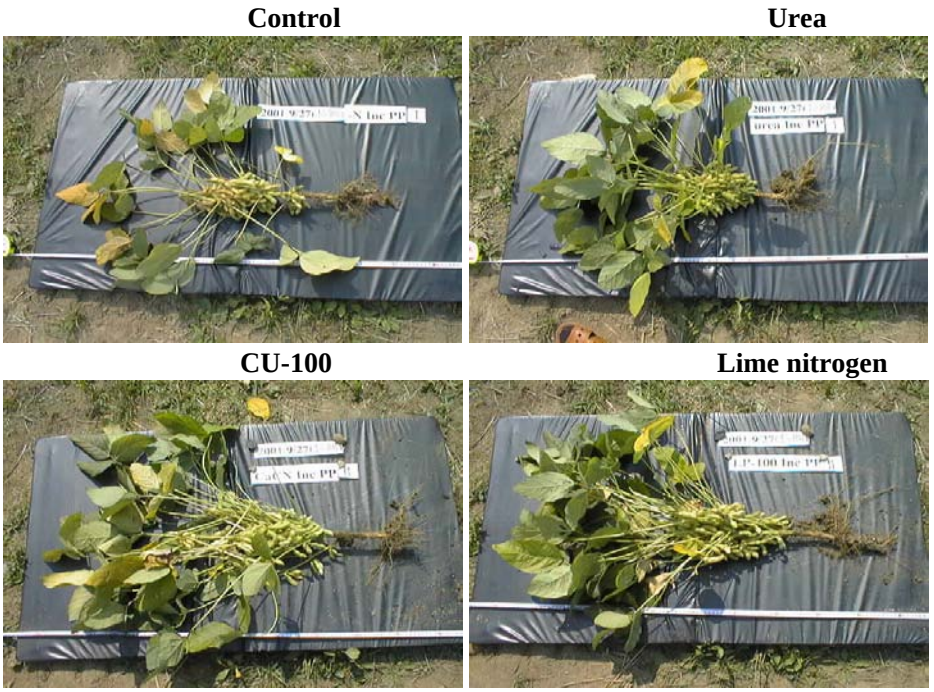


Figure 76. Effect of deep placement of urea, coated urea or lime nitrogen and paper pot with inoculation paper pot transplantation in Nagaoka. From Tewari et al. 2003

Table 4. Estimation of the amount of N originating from various N sources based on the ^{15}N dilution method.

Line	Deep Plancement	Ndfa (g/plant^{-1})	Ndfs (g/plant^{-1})	Ndff (g/plant^{-1})	Total N (g/plant^{-1})
Enrei	AS	2.24 b	0.78 c	0.09 c	3.12 b
	U	2.32 b	1.07 b	0.23 b	3.62 b
	CU	2.82 b	1.05 b	0.41 a	4.28 ab
	LN	3.61 a	1.34 a	0.48 a	5.43 a

AS; ammonium sulfate, U; urea, CU; coated urea, LN; lime nitrogen. Means followed by the same letter are not significantly different by 5% level. (From Tewari *et al.* 2007)

A significant increase in seed yield was observed in the IPP and DT methods compared with the NIPP method (Tewari *et al.* 2002, 2003, 2004a), suggesting that the inoculation of efficient strains such as *Bradyrhizobium japonicum* USDA110 might have improved soybean seed production. The promotive effect

of inoculation may be due to the low density of the bradyrhizobial population in the field as in the reclaimed field.

Minagawa et al. (1997) used the *gus* marked *Bradyrhizobium japonicum* strain for estimation of inoculated strain number in soil. The proliferation and mobility of inoculated *gus*-strain was examined in a rhizobox containing various soil types (Table 5). At 25 days after inoculation, rhizobial population in rhizobox increased 1218 times in Nagakura soil (alluvial soil), 538 times in Nakazawa soil (volcanic ash soil), 513 times in Sonoki soil (alluvial soil), 173 times in Ikarashi soil (sandy dune soil), and 98 times in vermiculite. Soybean cultivion had been frequently repeated in Nagakura and Nakazawa fields by rotation between paddy rice and soybean. Soybean cultivation was absent for a long time in Sonoki and Ikarashi fields. The indigenous rhizobium population per one g soil estimated by MPN (most probable number) method was 5.8×10^5 cells in Nagakura, 3.1×10^5 cells in Nakazawa, 1.7×10^4 cells in Sonoki, 8 cells in Ikarashi.

The effectiveness of paper pot inoculation in the field with high rhizobial density may be due to the difficulty for indigenous bradyrhizobia to infect the upper parts of the roots, by increasing the populations of efficient strain USDA110 during seedling nursery. As a result the infection rate of USDA 110 might increase and ultimately N_2 fixation became higher. In addition, the inoculation of uptake hydrogenase-positive (hup^+) strain USDA110 promoted soybean growth compared with the uptake hydrogenase-negative (hup^-) strains. Based on the fingerprint analysis of repeated sequences of genes, the incidence of *Bradyrhizobium japonicum* hup^+ was dominant in the Nagakura field compared with other sites in Japan (Minamisawa et al 1999). At the Nagakura site where the bradyrhizobia population appeared to be relatively abundant, the inoculation of USDA110 was associated with a promotive effect. Therefore, the inoculation practice may contribute to enhancing the soybean seed yield in the fields where a high indigenous bradyrhizobium population had been established.

6. Analysis of the Promotive Effect of Deep Placement of Slow Release N Fertilizers on Growth and Seed Yield of Soybean by ^{15}N Analysis

The effect of deep placement of ammonium sulfate (AS), urea (U), coated urea (CU) and lime nitrogen (LN) on the N origin was investigated by ^{15}N dilution method (Tewari et al. 2005). Deep placement of ^{15}N labeled fertilizers ($100 kgN\ ha^{-1}$) was applied in converted paddy field in the same field as above

(Nagaoka). Soybean plants cv. Enrei and the non-nodulated isogenic line En1282 were planted. Whole plants were sampled at maturing stage, and ^{15}N abundance and N concentration in each part were analyzed. The evaluation of Ndfa, Ndfs and Ndff was conducted by ^{15}N dilution method using En1282 as a reference plant as shown in Table 4. In all the treatment, non-nodulated line exhibited only 36-40% of total dry weight and 16-30% of total N accumulation compared with the nodulated line Enrei, due to N deficiency associated with the lack of nitrogen fixation. The value of the seed weight per plant of Enrei was highest in LN (73g) followed by CU (63g), U (47g), AS (37g) and control without deep placement (26g). The value of Ndfa per plant estimated by ^{15}N dilution method was higher in LN (3.6g) and CU (2.8g) than in U (2.3g) and AS (2.3g) treatments. The recovery rate of fertilizer N was higher in LN (43%) and CU (36%) than U (21%) and AS (21%) treatments. These results confirmed that deep placement of both LN and CU is effective to improve soybean growth and seed yield by promoting nitrogen fixation by root nodules.

Table 5. Characters of soils used for rhizobial proliferation and infection.

Location	Soil type	pH (H_2O)	CEC (meq 100 g^{-1})	Total N (%)	Available N (mg 100 g^{-1})	Available P (mg 100 g^{-1})
Nagakura	loam	6.52	27.36	0.08	3.49	20.03
Nakazawa	light clay	6.50	34.86	0.23	6.34	32.67
Sonoki	clay loam	6.64	21.64	0.13	12.85	57.10
Ikarashi	loamy sand	6.75	4.60	0.03	2.04	7.28
Vermi-culite	-	6.78	64.73	0.00	0.56	2.10

(From Minagawa *et al.* 1997)

To investigate the utilization of N from LN compared with CU, soybean plants were periodically sampled with deep placement of ^{15}N labeled LN and CU (Tewari et al 2007). Figure 77 shows the absorption pattern of labeled N from LN and CU. The N absorption was initially lower with LN than CU at the R3 and R5 stages, but the absorption was from LN exceeded CU at R7 stage. The recovery rate was 70% in LN and 61% in CU.

The daily N_2 fixation activity and N absorption rate was calculated by a simple relative ureide method (Figure 78). Average daily N assimilation rate in which N from N_2 fixation plus N from N absorption was relatively low until 61 DAS (R1 stage). Daily N_2 fixation activity from planting to R1 was higher in CU than Cont and LN treatments, although N absorption was high in LN during the

same period. From 61 to 102 DAS (from R1 to R5 stage), both the nitrogen absorption rate and N_2 fixation activity were shown to be high in LN and CU treatments, in comparison to Cont treatment. In all the treatments, the higher N_2 fixation activity was found during R3 and R5 stages with LN (630 mg m^{-2} d^{-1}), CU (616 mg m^{-2} d^{-1}), and Cont (464 mg m^{-2} d^{-1}) treatments. The N_2 fixation activity declined during the maturation stage (102-130 DAS) (R5-R7) in all the treatments. The distribution patterns of labeled N from LN and CU were similar among organs (Figure 79).

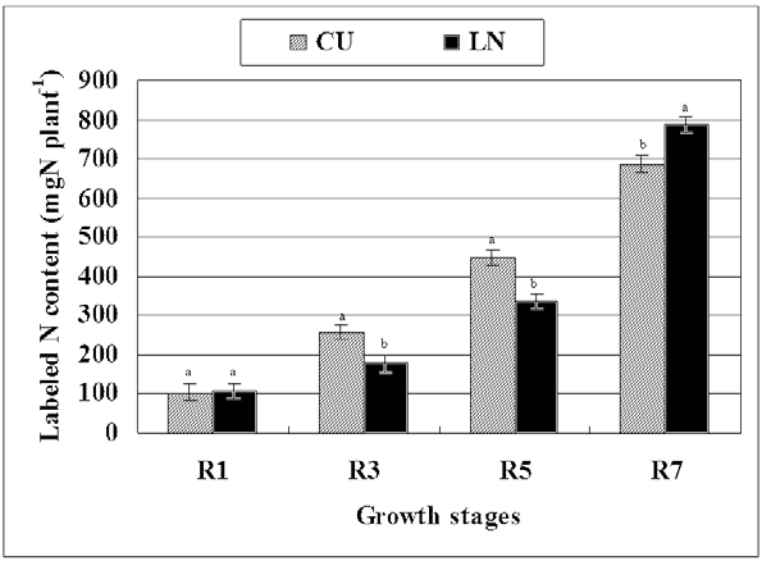


Figure 77. Changes in N recovery from coated urea and lime nitrogen with deep placement in the rotated paddy field in Nagaoka. From Tewari et al. 2003

The seed yield per plant was 37 g (Cont), 67 g (CU), and 71 g (LN), which are equivalent to 3.2 t, 5.8 t and 6.2 t per hectare respectively. The results of the naked eye examination of the seed quality are presented in Figure 80. The percentage of good seeds were 58% (Cont), 62% (CU), and 65% (LN), and thus the effect of deep placement of LN and CU on improving visual quality could be confirmed from this result. It can also be seen that there was a reduction in bad seeds, especially side wrinkle, whose percentage was slightly reduced by LN (17%) and CU (19%) treatment, compared to Cont (26%).

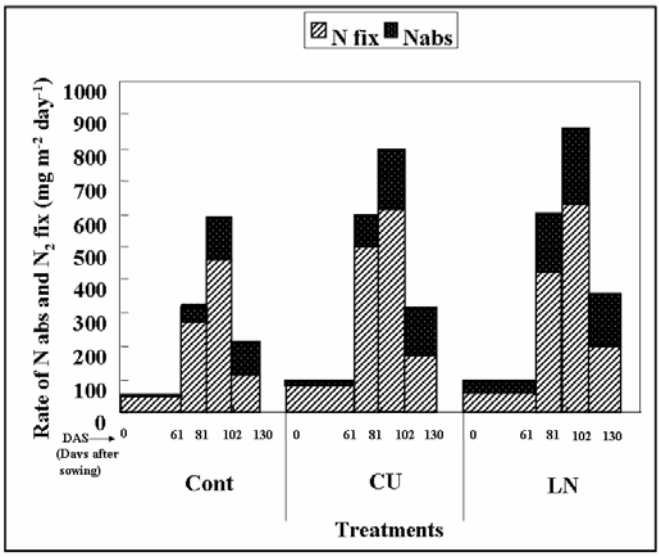


Figure 78. Changes in N_2 fixation activity and N absorption rate with deep placement of coated urea and lime nitrogen in the rotated paddy field in Nagaoka. From Tewari et al. 2007

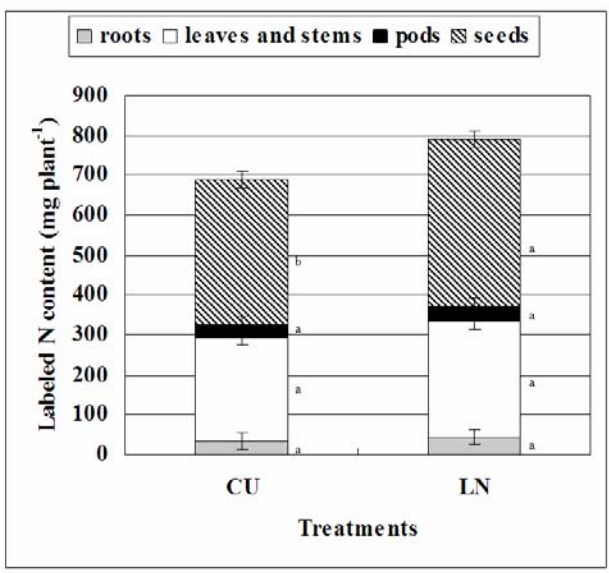


Figure 79. Distribution of labeled N at R7 stage from deep placement of coated urea and lime nitrogen in the rotated paddy field in Nagaoka..

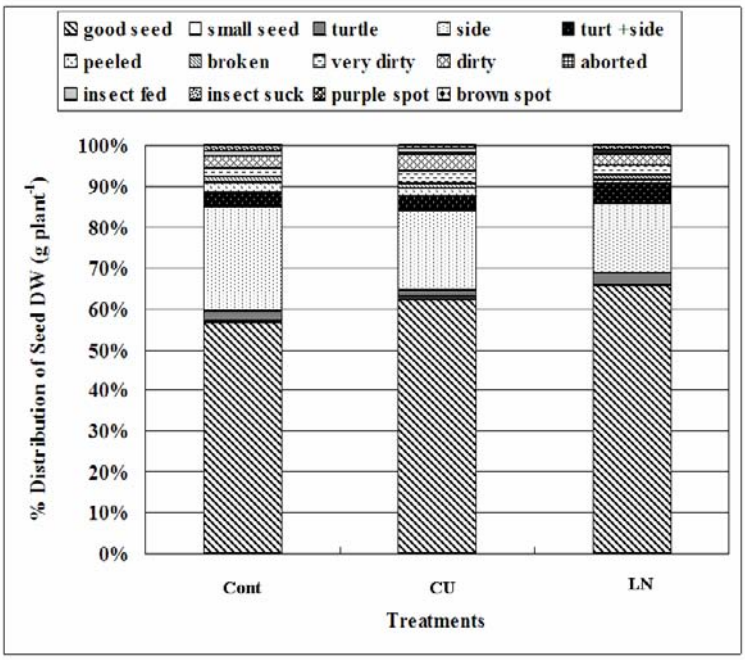


Figure 80. Seed quality with deep placement of coated urea and lime nitrogen in the rotated paddy field in Nagaoka. From Tewari et al. 2007

7. PROMOTIVE EFFECTS OF DEEP PLACEMENT OF LIME NITROGEN ON SEED YIELD.

The efficient supply of N after flowering and non-depressing or promotive effect on N_2 fixation activity may account for the high seed yields of the soybean plants with deep placement of $CaCN_2$ and CU-100. The yield increase by deep placement of CU-100 and $CaCN_2$ was mainly due to the increase in the pod number per plant. It has been suggested that optimum vegetative growth and sufficient nutrient supply is necessary to obtain an appropriate pod setting number. The number of total nodes includes the nodes of the main stem and branches is also important to increase pod number. The node number of the main stem is determined during the early vegetative growth period. Therefore, the increase of the seed yield could be achieved through the increase of the number of branches and pods per node by management practices. The deep placement of CU-100 as well as $CaCN_2$ contributed to the increase of the total node number in the

branches and the pod number per node, leading to the increase of seed yield. Based on the results obtained here, the deep placement of slow release N fertilizers on promotive effect of N_2 fixation may have enabled to maintain the supply of N to the shoot. Consequently, this condition may prevent flower and pod shedding due to nutrient competition or stress during the transition from the vegetative to the reproductive growth, which may account for the good yield obtained with both the $CaCN_2$ and CU-100 treatments. The number of pods increased by deep placement at the early reproductive stage. Thereafter, the abundant photoassimilate and continuous higher N supply could fill the pods during the seed growth period, which may account for the fact that the N content in seeds with $CaCN_2$ as well as CU-100 was also higher than that in the control and urea treatments.

Generally, N fertilization is known to depress nodulation and the N_2 fixation activity. The present results suggested that the deep placement of $CaCN_2$ as well as CU-100 alleviated the depression of nodulation and N_2 fixation. The continuous supply of a low level of ammonium or nitrate may be beneficial to keep leaves active over a long period of time, because the absorbed N tended to be primarily translocated to the leaves and then re-exported to the other growing parts (Ohyama 1983, Ohyama and Kawawi 1983) Moreover, the non-depressing effect of deep placement of CU-100 and $CaCN_2$ on N_2 fixation may be due to the fact that the inhibitory effect of NO_3^- (major form of absorbed N) is restricted to the root system, which is directly in contact with NO_3^-. It can be considered that a large number of active nodules are distributed in the surface layers of soil due to the adequate supply of N_2 and O_2 by diffusion through soil from air. Since the N released from deep placement of CU-100 and $CaCN_2$ may be absorbed in the deep layers, the inhibitory effect on N_2 fixation of the nodules in the upper layers may be attenuated. As a result, deep placement of controlled release fertilizers (100 kgN ha^{-1} of 100 day type coated urea or lime nitrogen) into 20 cm depth from soil surface with basal dressing of ammonium sulfate (16 kgN ha^{-1}) in the plow layer enabled to increase the soybean seed yield by about 10-85 % over the conventional cultivation.

The mechanism of promotion of deep placement of lime nitrogen for soybean growth and seed yield is summarized in Figure 81.

a. Deep placement of lime nitrogen is hydrolyzed to urea, then to ammonium and carbondioxiside. The ammonium does not easily leach out from the fertilization sites at 20 cm depth. Dicyandiamide contained in lime nitrogen or formed in the soil from lime nitrogen depresses nitrification to prevent ammonium oxidation to nitrate. As a result, nitrate

leaching and dinitrification is reduced and the ammonium can be sustained in the deep place for a long term.

b. The abundant supply of N in the lower layer promotes the lower root growth, and water and nutrients absorption activity and fertilized N is efficiently absorbed from the lower roots.
c. The abundant supply of N from lower part of roots promotes leaf growth and extends the photosynthetic activity until maturing stage. The leaf area and chlorophyll content was higher in the leaves of deep placement than those in control ones.
d. Abundant supply of photoassimilate to nodules supports the nodule growth and N_2 fixation activity for an extended period during maturing stage.
e. Continuous supply of N from nodules and roots with increased photoassimilate supply promotes seed yield without decreasing the quality.

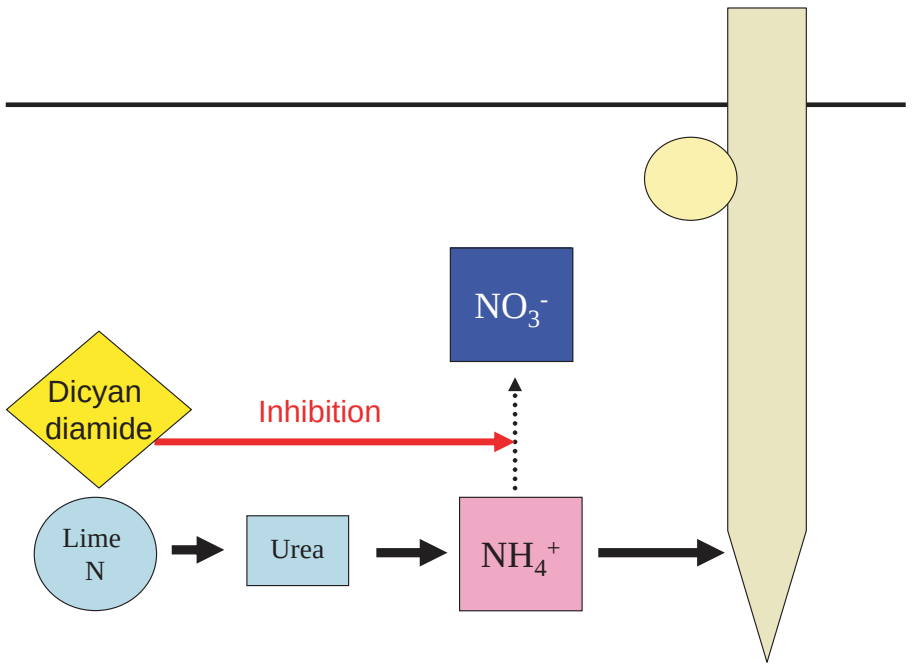


Figure 81. Fate N from deep placement of lime nitrogen in soil.

REFERENCES

Akao S and Kouchi H, 1992: A supernodulating mutant isolated from soybean cultivar Enrei. *Soil Sci. Plant Nutr.*, 38, 183-187

Arrese-Igor C, Gordon AJ, Minchin FR, and Denison RF, 1998: Nitrate entry and nitrite formation in the infected region of soybean nodules. *J. Exp. Bot.*, 49, 41-48

Atkins, CA, 1986. The legume:rhizobium symbiosis. Limitations to maximizing nitrogen fixation. *Outlook on Agric.*, 15, 128-134

Atkins CA, 1991: Ammonia assimilation and export of nitrogen from the legume nodule. In *Biology and Biochemistry of Nitrogen Fixation*, Elsevier Science Publishers, 293-319

Atwell BJ, 1992: Nitrate and ammonium as nitrogen sources for lupins prior to nodulation. *Plant Soil*, 139, 247-251

Bacanamwo M and Harper JE, 1996: Regulation of nitrogenase activity in *Bradyrhizobium japonicum*/soybean symbiosis by plant N status as determined by shoot C:N ratio. *Physiol. Plant.*, 98, 529-538.

Bacanamwo M and Harper JE, 1997: The feedback mechanism of nitrate inhibition of nitrogenase activity in soybean may involve asparagine and/or products of its metabolism. *Physiol. Plant.*, 100, 371-377.

Bergersen FJ 1965: Ammonia-an early stable product of nitrogen fixation by soybean nodules. *Aust. J. Biol. Sci*., 18, 1-9

Bergersen FJ, 1999: Delivery of N_2 to bacteroids in simulated soybean nodule cells: consideration of gradients of concentration of dissolved N_2 in cell walls, cytoplasm, and symbiosomes. *Protoplasma*, 206, 137-142

Board JE and Tan Q, 1995: Assimilatory capacity effects on soybean yield components and pod number. *Crop Sci*., 35, 846-851

Bohlool BB, Ladha JK, Garrity DP and George T, 1992: Biological nitrogen fixation for sustainable agricultre: A perspective. *Plant Soil*, 141, 1-11

Breteler H and Siegerist M, 1984: Effect of ammonium on nitrate utilization by roots of dwarf bean. *Plant Physiol*., 75, 1099-1103

Brown SM Oparka KJ, Sprent JI and Walsh KB, 1995: Symplastic transport in soybean root nodules. *Soil Biol. Biochem.*, 27, 387-399

Carroll BJ and Mathews A, 1990: Nitrate inhibition of nodulation in legumes. In: Gresshoff PM, ed. *Molecular Biology of Symbiotic Nitrogen Fixation.* Boca Raton, FL: CRC press, 159-180.

Carroll BJ, McNeil DL and Gresshoff PM, 1985: A supernodulation and nitrate-tolerant symbiotic (*nts*) soybean mutant. *Plant Physiol.*, 78, 34-40

Catford J-G, Staehelin C, Lerat S, Piche Y and Vierheilig H, 2003: Suppression of arbuscular mycorrhizal colonization and nodulation in split-root systems of alfalfa after pre-inoculation and treatment with Nod factors. *J. Exp. Bot.*, 54, 1481-1487

Cheng X-G, Nomura M, Sato T, Fujikake H, Ohyama T and Tajima S, 1999: Effect of exogenous NH_4^+-N supply on distribution of ureide content in various tissues of alfalfa plants, *Medicago sativa*. *Soil Sci. Plant Nutr.*, 45, 921-927

Clark SE, Williams RW and Meyerowits EM, 1996: The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. *Cell* 89: 575-585

Coates JB, Medeiros JS, Thanh VH and Nielsen NC, 1985: Characterization of the subunit of β-conglycinin. *Arch. Biochem. Biophys.*, 243, 184-194

Crawford NM and Glass ADM, 1998: Molecular and physiological aspects of nitrate uptake in plants. *Trends in Plant Sci.*, 3, 389-395

Creason GL, Holowach LP, Thompson JF and Madison JT, 1983: Exogenous methionine depress level of mRNA for a soybean storage protein. *BBRC*, 117, 658-662

Crespi M and Galves S, 2000: Molecular mechanisms in root nodule development. *J. Plant Growth Regul*. 19,155-166

Dakora FD, Atkins CA and Pate JS, 1992: Effect of NO_3 on N_2 fixation and tirogenous solutes of xylem in two nodulated West African geocarpic legumes, Kersting's bean (*Macrotyloma geocarpum* L.) and Bambara groundnut (*Vigna subterranea* L.). *Plant Soil*, 140, 255-262

Deaker R, Roughley RJ and Kennedy IR, 2004: Legume seed inculation technology- a Review. *Soil Biol. Biochem.*, 36, 1275-1288

Delhon P, Gojon A, Tillard P and Passama L, 1995a: Diurnal regulation of NO_3^- uptake in soybean plants. I. Changes in NO_3^- influx, efflux, and N utilization in the plant during the day/night cycle. *J. Exp. Bot.*, 46, 1585-1594

Delhon P, Gojon A, Tillard P and Passama L, 1995b: Diurnal regulation of NO_3^- uptake in soybean plants. II. Relationship with accumulation of NO_3^- and asparagine in the roots. *J. Exp. Bot*., 46, 1595-1602

Delves AC, Higgins AV and Gresshoff PM, 1987: Shoot control of supernodulation in a number of mutant soybean. *Aust. J. Plant Physiol.*, 128, 473-478

Denarie J, Debelle F and Rosenberg C, 1992: Signaling and host range variation in nodulation, *Annu. Rev. Microbiol*., 46, 497-531

Fehr, W.R., Caviness, C.E., Burmood, D.T., and Pennington, J.S., 1971. Stage development description for soybean *Glycine max* (L.) Merill. *Crop Sci*., 11, 929-931.

Ferguson BJ and Mathesius U, 2003: Signaling interactions during nodule development, *J. Plant Growth Regulation*, 22,47-72

Fellows RJ, Egli DB and Leggett JE, 1978: A pod leakage technique for phloem translocation studies in soybean (*Glycine max* [L.] Merr.). *Plant Physiol*. 62, 812-814

Finean JA, Coleman R and Michell RH, 1984: *Membranes and their cellular Functions, 3rd* Ed., Blackwell Scientific Publications, Oxford.

FNCA Biofertilizer manual, 2006: ISBN 4-88911-301-0 c0550, http://www.fnca.mext.go.jp/ english/index.html

Fuchsman WH, Barton CR, Stein MM, Thomson JT and Willett RM, 1976: Leghemoglobin: Different roles for different components ? *BBRC*, 68, 387-392

Fujikake H, Yashima H, Sato T, Ohtake N, Sueyoshi K and Ohyama T, 2002: Rapid and reversible nitrate inhibition of nodule growth and N_2 fixation activity in soybean (*Glycine max* (L.) Merr.). *Soil Sci. Plant Nutr.*, 48, 211-217

Fujikake H, Tamura Y, Ohtake N, Sueyoshi K and Ohyama T, 2003a: Photoassimilate partitioning in hypernodulation mutant of soybean (*Glycine max* (L.) Merr.) NOD1-3 and its parent Williams in relation to nitrate inhibition of nodule growth. *Soil Sci. Plant Nutr.*, 49, 583-590

Fujikake H, Yamazaki A, Ohtake N, Sueyoshi K, Matsuhashi S, Ito T, Mizuniwa C, Kume T, Hashimoto S, Ishioka NS, Watanabe S, Osa A, Sekine T., Uchida H, Tsuji A and Ohyama T, 2003b: Quick and reversible inhibition of soybean root nodule growth by nitrate involves a decrease in sucrose supply to nodules. *J. Exp. Bot.*, 54, 1379-1388

Fujita, T and Shoji S, 1999: Meister: Controlled relrease fertilizer, Ed. Shoji S, Konno Printing Company Ltd., Sendai

Gan Y, Stulen I, Van Keulen H and Kuiper JC, 2003:Effect of N fertilizer top dressing at various reproductive stages on growth, N_2 fixation and yield of three soybean (*Glycine max* (L.) Merr.) genotype. *Field Crop Research*, 80,147-155

Gayler KR and Sykes GE, 1985: Effects of nutritional stress on the storage proteins of soybeans. *Plant Physiol.*, 78, 582-585

Gerahty N, Caetano-Anolles G, Joshi PA, and Gresshoff PM, 1992: Anatomical analysis of nodule development in soybean reveals an additional autoregulatory control point. *Plant Sci*., 85, 1-7

Giannakis C, Nicholas DJD and Wallace W, 1988: Utilization of nitrate by bacteroids of Bradhyrhizobium japonicum in the soybean root nodule. *Planta*, 174, 51-58

Gibson AH and Harper JE, 1985: Nitrate effect on nodulation of soybean by *Bradyrhizobium japonicum*. *Crop Science*, 25, 497-501.

Gordon AJ, Skøt L, James CL and Minchin FR, 2002: Short-term metabolic response of soybean root nodules to nitrate. *J. Exp. Bot.*, 53, 423-428.

Gremaud MF and Harper JE, 1989: Selection and initial characterization of partially nitrate tolerant nodulation mutants of soybean. *Plant Physiol.*, 89, 169-173

Gundlach H, Muller MJ, Kutchan TM and Zenk MH, 1992: Jasmonic acid is a signal transfucer in elicitor-induced plant cell cultures. *Proc. Natl. Acad. Sci. U.S.A.*, 89, 2389-2393

Haga KI and Sodek L, 1987: Utilization of nitrogen sources by immature soybean cotyledons in culture. *Ann. Bot*., 59, 597-601

Hansen AP, Yoneyama T and Kouchi H, 1992: Short-term nitrate effects on hydroponically-grown soybean cv. Bragg and its supernodulating mutant. *J. Exp. Bot.*, 43, 1-7

Harderson G, 1993: Method for enhancing symbiotic nitrogen fixation. *Plant Soil*, 152, 1-17

Harper JE, 1974: Soil and symbiotic nitrogen requirements for optimum soybean production. *Crop Sci.*, 14, 255-260.

Harper JE and Gibson AH, 1984: Differential nodulation tolerance to nitrate among loegume species, *Crop Sci.*, 14, 255-260

Harper JE. 1987: *Nitrogen metabolism. Soybeans: Improvement, production and Uses.* 2nd ed. *Agronomy Monograph* no.16. ASA-CSSA-SSSA., 497-533.

Hayati R, Egli DB and Crafts-Brandner SJ, 1995: Carbon and Nitrogen supply during seed filling and leaf senescence in soybean. *Crop Sci.*, 35, 1063-1069

Herridge DF and Danso SKA, 1995: Enhancing crop legume N_2 fixation through selection and breeding. *Plant and Soil*, 174, 51-82

Herridge D, and Rose I, 2000: Breeding for enhanced nitrogen fixaion in crop legumes. *Field Crop Research*, 65, 229-248

Hirsch A, and Fang Y, 1994: Plant hormones and nodulation: what's the connections?, *Plant Molecular Biology*, 26, 5-9

Hirsch AM and Kapulnik Y, 1998: Singal transduction pathways in mycorrhizal associations: comparisons with the rhizobium-legume symbiosis. *Fungal Genetics and Bilology*, 23, 205-212

Holowach LP, Thomson JF and Madison JT, 1984: Effects of exogenous methione on storage protein composition of soybean cotyledons cultured in vitro. *Plant Physiol*., 74, 576-583

Holowach LP, Madison JT and Thomson JF, 1986: Studies on the mechanism of regulation of the mRNA level for a soybean storage protein subunit by exogenous L-methionine. *Plant Physiol*., 80, 561-567

Hoshi S, 1982: Nitrogen fixation, growth and yield of soybean. In*: Nitrogen fixation in Root Nodules*, Ed. Japanese Society of Soil Science and Plant Nutrition, Hakuyusha Publishers, Japan, pp 5-33.

Ito S, Ohtake N, Sueyoshi K and Ohyama T, 2006a: Allocation of photosynthetic products in soybean during the early stages of nodule formation. *Soil Sci. Plant Nutr.*, 52,438-443

Ito S, Ohtake N, Sueyoshi K and Ohyama T, 2006b: Allocation of photosynthetic products in hypernodulation mutant of soybean NOD1-3 in the early stages of nodule formation. *Bull. Facul. Agric. Niigata Univ.*, 59: 33-38

Ito S, Ohtake N, Sueyoshi K and Ohyama T. 2006c: Characteristics of initial growth of hypernodulation soybean mutants, NOD1-3, NOD2-4 and NOD3-7, and their parent cv. Williams. *Bull. Facul. Agric. Niigata Univ.*, 59: 39-43

Ito S, Ohtake N, Sueyoshi K and Ohyama T. 2007: Characteristics of initial growth of hypernodulation soybean mutants, NOD1-3, NOD2-4 and NOD3-7, affected by inoculation of bradyrhizobia and nitrate supply. *Soil Sci. Plant Nutr*., 53: 66-71

Ito S, Kato T, Ohtake N, Sueyoshi K and Ohyama T. 2008: The autoregulation of nodulation mechanism is related to leaf development, *Plant Cell Physiol.*, 49: 121-125

Kanayama Y and Yamamoto Y, 1990a: Inhibition of nitrogen fixation in soybean plants supplied with nitrate I. Nitrite accumulation and formation of nitrosylleghemoglobin in nodules. *Plant Cell Physiol.*, 31, 341-346

Kanayama Y and Yamamoto Y, 1990b: Inhibition of nitrogen fixation in soybean plants supplied with nitrate II. Accumulation and properties of nitrosylleghemoglobin in nodules. *Plant Cell Physiol.,* 31, 207-214

Kanayama Y and Yamamoto Y, 1990c: Inhibition of nitrogen fixation in soybean plants supplied with nitrate III. Kinetics and formation of nitrosylleghemoglobin and of the inhibition of formation of oxyleghemoglobin. *Plant Cell Physiol.,* 31, 603-608

Kanayama Y and Yamamoto Y, 1990d: Effects of nitrate on nucleotide levels in soybean nodules. *Plant Cell Physiol.,* 31, 893-895

Keyser HH and Li F, 1992: Potential for increasing biological nitrogen fixation in soybean. *Plant Soil*, 141,119-135

Konno, S, 1976. Possibility of increasing the productivity of soybean, in *Perspective in Technical Development of Self-Suport of Food*, (Kondo, Y. Ed. Norin Tokei Kyokai, Tokyo, Japan) 121-134

Kouchi H, Shimomura K, Hata S, Hirota A, G-J Wu, Kumagai H, Tajima S, Suganuma N, Suzuki A, Aoki T, Hayashi M, Yokoyama T, Ohyama T, Asamizu E, Kuwata C, Shibata D and Tabata S, 2004: Large-scale analysis of gene expression profiles during early stages of root nodule formation in a model legume, *Lotus japonicus*. *DNA Research,* 11, 263-274

Krusell L, Madsen LH, Sato S, Aubert G, Genua A, Scyglowski K, Duc G, Kaneko T, Tabata S, de Bruijn F, Pajuelo E, Sandal N and Stougaard J, 2002: Shoot control of root development and nodulation is mediated by a receptor-like kinase. *Nature* 420: 422-426

Kubo H, 1939: Uber das Hamoprotein aus den Wurzelknollchen von Leguminosen, *Acta Phytochimica*, 11, 195-200

Kushizaki M, Ishizuka J and Akamatsu F, 1964: Physiological studies on the nutrition of soybean plants. 2. Effect of nodule formation on nitrogenous constituents of soybeans, *J. Sci. Soil Manure, Japan*, 35, 323-327

Layzell DB, 1990: N_2 fixation, NO_3^- reduction and NH_4^+ assimilation. In Plant Physiology, Biochemistry and Molecular Biology, Ed. Dennis, DT, and Turpin, DH, Longman Scientific Technology, (Essex, UK)

Lee KH and LaRue TA, 1992a: Ethylene as a possible mediator of light-and nitrate-indulced inhibition of nodulation of *Pisum sativum* L. cv Sparkle. *Plant Physiol.,* 100, 1334-1338

Lee KH and LaRue TA, 1992b: Exogenous ethylene nodulation of *Pisum sativum* L. cv Sparkle. *Plant Physiol.,* 100, 1759-1763

Li Y. et al., 2001: Supply of O_2 regulates demand for O_2 and uptake of malate by N_2-fixing bacterioids from soybean nodules. *Microbiology*, 147. 663-670

Ligero F, Caba JM, Lluch C and Oliveres J, 1991: Nitrate inhibition of nodulation can be overcome by the ethylene inhibitor aminoethoxyvinylglycine. *Plant Physiology*, 97, 1221-1225.

Limpens E and Bisseling T, 2003: Signaling in symbiosis. *Curr. Opin. Plant Sci.*, 6, 343-350

Liu, X, and Herbert SJ, 2002: Fifteen years of research examining cultivation of continuous soybean in northeast China, *Field Crop Research*, 79, 1-7

Loake G and Grant M, 2007: Salicylic acid in plant defence-the players and protagonists. *Curr. Opinion in Plant Biol*., 10, 466-472

Lum MR and Hirsch AM, 2003: Roots and their symbiotic microbes: Strategies to obtain nitrogen and phosphorus in a nutrient-limiting environment. *J. Plant Growth Regul.*, 21, 368-382

Malamy J, Carr JM, Klessig DF and Raskin I, 1990: Salicylic acid; A likely endogenous signal in the resistance response of tobacco to viral infection. *Science*, 250, 1002-1004

Masuda R, Sugimoto T, Shiraishi N, OhyamaT and Oji Y, 2003: Ureide and amino acids in xylem sap of soybean (*Glycine max* L.) are affected by both nodulation and nitrogen supply form soil. *Soil Sci. Plant Nutr.*, 49, 185-190

Matsumoto T, Yamamoto Y and Yatazawa M, 1975: Role of root nodules in the nitrogenous nutrition of soybean 1. Fluctuation of allantoin and some other plant constituents in the growing period. *J. Sci. Soil Manure, Japan*, 46, 471-477

Meixner C, Vegvari G, Ludwig-Muller J, Gagnon H, Steinkellner S, Staaehelin C, Gresshoff P, Vierheilig H, 2007: Two defined alleles of the LRR receptor kinase *GmNARK* in supernodulating soybean gonvern differing autoregulation of mycorrhization. *Physiol. Plant*., 130, 261-270

Metraux JP, Signer H, Ryals J, Ward E, Wyss-Benz M. Gaudin J, Raschdorf K, Schimid E, Blum W and Inverardi B, 1990: Increase in calicylic acid at the onset of systemic acquired resistance in cucumber, *Science*, 250, 1004-1006

Minagawa R, Isoda K, Ohtake N, Yamada S, Ikarashi T, Minamisawa K and Ohyama T, 1997: Estimation of gus marked Bradyrhizobial number by phenol extraction of GUS metabolite. *J. Sci. Soil Manure, Japan*, 68, 148-155

Minamisawa K, Nakatsuka Y and Isawa T, 1999: Diversity and field site variation of indigenous populations of soyban bradyrhizobia in Japan by fingerprints with repeated sequences RSα and RSβ *FEMS Microbiol. Ecol.*, 29,171-178

Mizukoshi K, Nishiwaki T, Ohtake N, Minagawa R, Ikarashi T, and Ohyama T, 1995: Nitrate transport pathway into soybean nodules traced by tungstate and $^{15}NO_3^-$. *Soil Sci. Plant Nutr.*, 41,75-88

Nakasathien S, Israel DW, Wilson RF and Kwanyuen P, 2000: Regulation of seed protein concentration in soybean supra-optimum nitrogen supply. *Crop Sci*., 40, 1277-1284

Neo HH and Layzell DB, 1997: Phloem glutamine and the regulation of O_2 diffusion in legume nodules. *Physiologia. Plantarum* 113, 259-267.

Nishimura R, Hayashi M, Wu GJ, Kouchi H, Imaizumi-Anraku H, Murakani Y, Kawasaki S, Akao S, Ohmori M, Nagasawa M, Harada K and Kawaguchi M, 2002: HAR1 mediates systemic regulation of symbiotic organ development. *Nature* 420: 426-429

Nishiwaki T, Mizukoshi K, Kobayashi K, Ikarashi T, and Ohyama T, 1995: Effect of culture medium compositions on nodulation, nitrogen fixation and growth of soybean plant. *Bull. Facul. Agric. Niigata Univ.*, 47, 73-83

Nishiwaki T and Ohyama T, 1995: Purification of soybean leghemoglobin and measurement of the ratio of two major leghemoglobin components, Lba and Lbc. *Bull. Facul. Agric. Niigata Univ*., 47, 85-92

Nishiwaki T, Mizukoshi K, Minagawa R, Sato T, Ohtake N, Ikarashi T, and Ohyama T, 1996: Effect of plant population and NO_3^- supply on soybean nodulation. Bull. Facul. Agric. Niigata Univ., 48, 71-79

Nishiwaki T, Sato T, Yamashita H, Ikarashi T, Ohyama T, Harper JE, Akao S and Kouchi H 1997: Changes in concentration of leghemoglobin components in hypernodulation mutants of soybean. *Soil Sci. Plant Nutr.*, 43, 1091-1096

Ofosu-Budu KG, Noumura K and Fujita K, 1995: N_2 fixation, N transfer and biomass production of soybean cv. Bragg or its supernodulating nts1007 and sorghum mixed-cropping at two rates of N fertilizer. *Soil Biol. Biochem.*, 24, 311-317

Ohtake N, Nishiwaki T, Mizukoshi K, Chinushi T, Takahashi Y and Ohyama T, 1994: Lack of β-subunit of β–conglycinin in non-nodulating isolines of soybean. *Soil Sci.Plant Nutr*., 40,345-349

Ohtake N, Nishiwaki T, Mizukoshi K, Minagawa R, Takahashi Y, Chinushi T and Ohyama T, 1995: Amino acid composition in xylem sap of soybean related to the evaluation of N_2 fixation by the relative ureide method. *Soil Sci.Plant Nutr*., 41, 95-102.

Ohtake N, Suzuki M, Takahashi Y, Fujiwara T, Chino M, Ikarashi T and Ohyama T, 1996: Differential expression of β–conglycinin genes in nodulated and non-nodulating isolines of soybean. *Physiol. Plant*., 96,101-110

Ohtake N, Ikarashi Y, Ikarashi T and Ohyama T, 1997a: Distribution of mineral elements and cell morphology in nodulated and non-nodulated soybean seeds. Bull. Facul. Agric. Niigata Univ., 49, 93-101

Ohtake N, Yamada S, Suzuki M, Takahashi N, Takahashi Y, Chinushi T and Ohyama T, 1997b: Regulation of accumulation of β-subunit of β-conglycinin in soybean seeds by nitrogen. *Soil Sci.Plant Nutr*., 43, 247-253

Ohtake N, Kawachi T, Sato A, Okuyama, I, Fujikake H, Sueyoshi K and Ohyama T, 2001a: Temporary application of nitrate to nitrogen-deficient soybean plants at the mid- to late-stages of seed development increased the accumulation of the β-subunit of β–conglycinin, a major seed storage protein. *Soil Sci.Plant Nutr*., 47, 195-203

Ohtake N, Sato A, Fujikake H, Sueyoshi K and Ohyama T, Ishioka NS, Watanabe S, Osa A, Sekine T., Matsuhashi S, Ito T, Mizuniwa C, Kume T, Hashimoto S, Uchida H and Tsuji A, 2001b: Rapid N transport to pods and seeds in N-deficient soybean plants. *J. Exp. Bot.*, 52, 277-283

Ohtake N, Kawachi T, Okuyama, I, Fujikake H, Sueyoshi K and Ohyama T, 2002: Effect of short-term application of nitrogen on the accumulation of β-subunit of β–conglycinin in nitrogen-starved soybean (*Glycine max* L.) developing seeds. *Soil Sci.Plant Nutr*., 48, 31-41

Ohyama T and Kumazawa K, 1978: Incorporation of ^{15}N into various nitrogenous compounds in intact soybean nodules after exposure to $^{15}N_2$ gas. *Soil Sci.Plant Nutr*., 24, 525-533.

Ohyama T and Kumazawa K 1979a: Assimilation and transport of nitrogenous compounds originated from $^{15}N_2$ fixation and $^{15}NO_3^-$ absorption. *Soil Sci.Plant Nutr*., 25, 9-19

Ohyama T and Kumazawa K 1979b: Assimilation and transport of nitrogenous compounds originating from $^{15}N_2$ fixation and $^{15}NO_3^-$ absorption. *Stable Isotopes, Proceedings of the third International Conference.*327-335 (Academic Press)

Ohyama T and Kumazawa K, 1980a: Nitrogen assimilation in soybean nodules I. The role of GS/GOGAT system in the assimilation of ammonia produced by N_2 fixation. *Soil Sci.Plant Nutr*., 26, 109-115

Ohyama T and Kumazawa K 1980b: Nitrogen assimilation in soybean nodules II. $^{15}N_2$ assimilation in bacteroid and cytosol fractions of soybean nodules. *Soil Sci.Plant Nutr*., 26, 205-213

Ohyama T and Kumazawa K 1980c: Nitrogen assimilation in soybean nodules III. Effect of rhizaosphere pO_2 on the assimilation of $^{15}N_2$ in nodules attatched to intact plants. *Soil Sci.Plant Nutr*., 26, 321-324

Ohyama T, Owa N, Fujishima Y and Kumazawa K, 1981a: Nitrogen assimilation in soybean nodules IV. Allantoin formation and transport in relation to supply with various forms of combined nitrogen. *Soil Sci.Plant Nutr*., 27, 55-64

Ohyama T and Kumazawa K, 1981b: Nitrogen assimilation in soybean nodules V. Possible pathway of allantoin synthesis in soybean nodules. *Soil Sci.Plant Nutr*., 27, 111-114

Ohyama T and Kumazawa K, 1981c: A simple method for the preparation, purification and storage of $^{15}N_2$ gas for biological nitrogen fization studies. *Soil Sci.Plant Nutr*., 27, 263-265

Ohyama T and Kumazawa K 1982: Emission spectrometric ^{15}N analysis of amino acids. *RADIOISOTOPES*, 31, 212-221

Ohyama T, 1983: Comparative studies on the distribution of nitrogen in soybean plants supplied with N_2 and NO_3^- at the pod filling stage. *Soil Sci.Plant Nutr*., 29, 133-145.

Ohyama T and Kawai S, 1983: Nitrogen assimilation and transport in soybean leaves: Investigation by petiole girdling treatment. *Soil Sci.Plant Nutr*., 29, 227-231.

Ohyama T, 1984. Comparative studies on the distribution of nitrogen in soybean plants supplied with N_2 and NO_3^- at the pod filling stage. II. Assimilation and transport of nitrogenous constituents. *Soil Sci.Plant Nutr*., 30, 219-229.

Ohyama T and Kumazawa K, 1987: Characteristics of nitrate respiration of isolated soybean bacteroids. *Soil Sci. Plant Nutr.*, 33, 69-78

Ohyama T, Saito K and Kato N, 1989a: Assimilation and transport of nitrate, nitrite, and ammonia absorbed by nodulated soybean plants. *Soil Sci.Plant Nutr*., 35, 9-20.

Ohyama T, Saito K and Kato N, 1989b: Diurnal rhythm in nitrate absorption by roots of soybeans (*Glycine max*). *Soil Sci.Plant Nutr*., 35, 33-42.

Ohyama T, Kato N and Saito K, 1989c: Nitrogen transport in xylem of soybean plant supplied with $^{15}NO_3^-$. *Soil Sci.Plant Nutr*., 35, 131-137.

Ohyama T and Harper JE, 1991: Effects of shoot removal on N_2 fixation and assimilation in nodulateion mutant and wild-type soybean. *Soil Sci. Plant Nutr*., 37, 471-476.

Ohyama T, Takahashi Y, Chinushi T, and Nakano T, 1992: Evaluation of N_2 fixation activity and nitrogen absorption rate in field grown soybean plants by simple relative ureide method. *Agriculture and Horticulture* (Nogyo oyobi Engei), 67, 1157-1164.

Ohyama T, Mizukoshi K and Nishiwaki T, 1993a: Distribution of ureide originated from nodules attached to the upper roots and nitrate derived from lower roots in soybean plants cultivated by double piled pots. *Bull. Facul. Agric. Niigata Univ.*, 45, 107-116.

Ohyama T, Nicholas JC and Harper JE, 1993b: Assimilation of $^{15}N_2$ and $^{15}NO_3^-$ by partially nitrate-tolerant nodulation mutants of soybean. *J. Exp. Bot.*, 44, 1739-1747

Ohyama T, Ohtake N, Nishiwaki T. Mizukoshi K, Minagawa R, Kobayashi K, Chinushi T and Takahashi Y, 1994a: Effect of N fertilization on seed quality and xylm transport forms in nodulating and non-nodulating soybean isolines *Bull. Facul. Agric. Niigata Univ.*, 46, 57-70.

Ohyama T, Ohtake N, Chinushi T and Takahashi Y, 1994b: Effect of deep placement of coated urea slow release nitrogen fertilizer on chemical composition of soybean seeds. *Jpn J. Soil Sci.Plant Nutr*., 65, 41-47

Ohyama T., Tewari K, Latif SA, Ruamrungsri S, Komiyama S, Ito S, Yamazaki A, Sueyoshi K and Ohtake N, 2004: Direct Analysis of ^{15}N Abundance of Kjeldahl Digested Solution by Emission Spectrometry. *Bull. Facul. Agric. Niigata Univ*., 57, 43-50

Paek NC, Imsande J, Shoemaker RC and Shibles R, 1997: Nutritional control of soybean seed storage protein. *Crop Sci.*, 37, 498-503

Rainbird RM, Thorne JH and Hardy RWF, 1984: Role of amides, aminoacids and ureides in the nutrition of developing soybean seeds. *Plant Physiol.*, 74, 329-334

Rolfe BG and Gresshoff PM, 1988: Genetic analysis of legume nodule initiation. *Ann. Rev. Plant. Physiol. Plant Mo. Biol.*, 39, 297-319

Sato T, Yashima H, Harper JE, Akao S and Ohyama T 1997a: Nodule formation and N_2 fixation traits of the rooted-single leaf isolated from hypernodulating mutants and the wild type. *Jpn J. Soil Sci. Plant Nutr*., 68, 444-447

Sato T, Nishiwaki T, Ohtake N and Ohyama T 1997b: Determination of concentration of soybean nodule leghemoglobin components by capillary electrophoresis. *Jpn J. Soil Sci. Plant Nutr.*, 68, 521-526

Sato T, Yashima H, Ohtake N, Sueyoshi K, Akao S, Harper JE and Ohyama T 1998: Determination of leghemoglobin components and xylem sap composition by capillary electrophoresis in hypernodulation soybean mutants cultivated in the field. *Soil Sci. Plant Nutr.*, 44, 635-645

Sato T, Yashima H, Ohtake N, Sueyoshi K, Akao S and Ohyama T 1999a: Possible involvement of photosynthetic supply in changes of nodule characteristics of hypernodulating soybeans. *Soil Sci. Plant Nutr.*, 45, 187-196

Sato T, Ohtake N, Ohyama T, Ishioka NS, Watanabe S, Osa A, Sekine T, Uchida H, Tsuji A, Matsuhashi S, Ito T and Kume T, 1999b: Analysis of nitrate absorption and transport in non-nodulated and nodulated soybean plants with $^{13}NO_3^-$ and $^{15}NO_3^-$. *Radioisotopes*, 48, 450-458.

Sato T, Nishiwaki T, Ohtake N, Sueyoshi K and Ohyama T 1999c: Non-involvement of ethylene action on the nitrate inhibition of nodulation in hypernodulation soybean mutant and its parent cv. Williams. *Bull. Fac. Agric. Niigata Univ.,* 51, 121-130

Sato T, Onoma N, Fujikake H, Ohtake N, Sueyoshi K and Ohyama T 2001: Changes in four leghemoglobin components in nodules of hypernodulating soybean (*Glycine max* [L] Merr.) mutant and its parent in the early nodule developmental stage. *Plant and Soil,* 237, 129-135

Sato T, Fujikake H, Ohtake N, Sueyoshi K, Takahashi T, Sato A and Ohyama T 2002: Effect of exogenous salicylic acid supply on nodule formation of hypernodulationg mutant and wild type of soybean. *Soil Sci. Plant Nutr.,* 48, 413-420

Sato S, Nakamura Y, Asamizu E, Isobe S and Tabata S, 2007: Genome sequencing and genome resources in model legumes. *Plant Physiol*., 144, 588-593

Schimidt JS, Harper JE, Hoffman TK and Bent AF, 1999: Regulation of soybean nodulation independent of ethylene signaling. *Plant Physiol.,* 119, 951-959.

Schubert KR, 1986: Products of biological nitrogen fixation in higher plants; Synthesis, transport, and metabolism. *Ann. Rev. Plant Physiol*., 37, 539-574

Schuller KA, Minchin FR and Gresshoff PM, 1988: Nitrogenase activity and oxygen diffusion in nodules of soybean cv. Bragg and a supernodulating mutant: effects of nitrate. *J. Exp. Bot.,* 39, 865-877.

Searle IR, Men AE, Laniya TS, Buzas DM, Iturbe-Ormaetxe I, Carroll BJ and Gresshoff PM, 2003: Long-distance signaling in nodulation directed by a CLAVATA1-like receptor kinase. *Science,* 299: 109-112

Senoo K, Kaneko M, Taguchi R, Murata J, Santasup C, Tanaka A and Obata H, 2002: Enhanced growth and nodule occupancy of red kidney bean and soybean inoculated with soil aggregate-based inoculant. *Soil Sci. Plant Nutr.,* 48, 215-259

Serraj R, Frangne N, Maeshima M, Fleurat-Lessard P and J-J Drevon, 1998: A γ-TIP cross-reacting protein is abundant in the cortex of soybean N_2-fixing nodules. *Planta,* 206, 681-684

Sharifi M and Zebarth BJ, 2006: Nitrate influx kinetic parameters of five potato cultivars during vegetative growth. *Plant Soil,* 288, 91-99

Shioya T, 1985: Abstract, 1985 Meeting, Farm Work Research Congress, Japan, 47

Shrihari PC, Sakamoto K, Inubushi K and Akao S, 2000: Interaction between supernodulating and non-nodulating mutants of soybean and two mycorrhizal fungi. *Mycorrhiza*, 10 (3), 101-106

Shuttuck-Eidens DM and Beachy RN, 1985: Degradation of β-conglycinin in early stage of soybean embryogenesis. *Plant Physiol.*, 76, 301-306

Silsbury JH, Catchpoole DW and Wallance W, 1986: Effects of nitrate and ammonium on nitrogenase (C_2H_2 reduction) activity of swards of subterranean clover, Trifolium subterraneum L. *Australian J. of Plant Physiol.*, 13, 257-273.

Song L, Carroll BJ, Gresshoff PM and Herridge DF, 1995: Field assessment of supernodulating genotypes of soybean for yield, N_2 fixation and benefit to subsequent crops. *Soil BIol. Biochem.*, 27, 563-569

Spaink HP, 1995: The molecular basis of infection and nodulation by rhizobia: The Ins and Outs of sympathogenesis. *Annu. Rev. Pytopathol.*, 33, 345-368

Spaink HP, 2000: Root nodulation and infection factors produced by rhizobial bacteria. *Ann. Rev. Microbiol.*, 54, 5257-288

Sprent JI, 1989: Which steps are essential for the formation of functional legume nodules ? *New Phytol.*, 111,129-153

Sprent JI and James EK, 2007: Legume Evolution: Where do nodules and mycorrhizae fit in ? *Plant Physiol.*, 144, 575-581

Stacey G, 1995: *Bradyrhizobium japonicum* nodulation genetics. *FEMS Microbiology Letters*, 127, 1-9

Stougaard J 2001: Genetics and genomics of root symbiosis. *Curr. Opin. Plant Biol.* 4, 328-335

Streeter JG, 1988: Inhibition of legume nodule formation and N_2 fixation by nitrate. *CRC Crit. Rev. Plant Sci.*, 7, 1-23

Suganuma T, Fujikake H, Ohtake N, Sueyoshi K and Ohyama T, 2001: Comparison of the growth and nitrogen fixation activity of the hypernodulatin soybean mutant NOD1-3 and its parent cv. Williams in field cultivation. *Bull. Facul. Agric. Niigata Univ.*, 53, 123-132

Suzuki A, Akune M, Kogiso M, Imagama Y, Osuki K, Uchiumi T, Higashi S, Han S, Yoshida S, Asami T and Abe M, 2004: Control of nodule number by the phytohormone abscisic acid in the roots of two leguminous species, *Plant Cell Physiol*., 45, 914-922

Tajima S, Nomura M and Kouchi H, 2004: Ureide biosynthesis in legume nodules,. *Frontiers in Bioscience*, 9, 1374-1381

Takahashi Y, Chinushi T, Nagumo Y, Nakano T and Ohyama T, 1991a: Effect of deep placement of controlled release nitrogen fertilizer (coated urea) on growth, yield and nitrogen fixation of soybean plants. *Soil Sci. Plant Nutr*., 37, 223-231.

Takahashi Y, Chinushi T, Nakano T, Hagino K, and Ohyama T, 1991b: Effect of placement of coated urea on root growth and rubidium uptake activity in soybean plants. *Soil Sci. Plant Nutr*., 37, 735-739.

Takahashi Y,Chinushi T, Nakano T and Ohyama T, 1992: Evaluation of N_2 fixation and N absorption activity by relative ureide method in field grown soybean plants with deep placement of coated urea. *Soil Sci. Plant Nutr*., 38, 699-708.

Takahashi Y, Chinushi T and Ohyama T, 1993a: Quantitative estimation of N_2 fixation and absorption rate in field grown soybean plants by relative ureide method. *Bull. Fac. Agric. Niigata Univ*., 45, 91-105.

Takahashi Y,Chinushi T, Nakano T and Ohyama T, 1993b: Behavior of fertilized N from top-dressed or deep placed coated urea in soil of soybean field. *Jpn J. Soil Sci. Plant Nutr.*, 64, 338-340

Takahashi Y,Chinushi T, Nakano T and Ohyama T, 1994: Yield components of soybean plants with deep placement of N fertilizer, related to high productivity. *J. Niigata Agric. Exp. Stn.*, 40, 7-15

Takahashi Y, 1995: Invention of fertilizer application by using controlled-release fertilizers. 3. Deep placement technique of coated urea fertilizer in soybean plants, *Jpn J. Soil Sci. Plant Nutr.*, 66, 277-285

Takahashi Y, Chinushi T and Ohyama T, 1995: Characteristics of growth and N nutrition of soybean affected by cool-summer damage in 1993 estimated by Rb/K ratio method and relative ureide method, *Jpn J. Soil Sci. Plant Nutr.*, 66, 127-132

Takahashi Y and Ohyama T, 1999: Technique for deep placement of coated urea fertilizer in soybean cultivation. *JARQ*, 33, 235-242.

Takahashi Y, Ohtake N, Hottori M, Nagumo Y and Ohyama T, 2006: Effect of basal side-dressing of various types of coated urea fertilizer on shoot growth, yield components and seed composition of soybean (*Glycine max* (L.) Merr.), *Soil Sci. Plant Nutr.*, 52, 264-273.

Tanaka A, Fujita K and Terasawa H, 1985: Growth and dinitrogen fixation of soybean root system affected by partial exposure to nitrate. *Soil Sci. Plant Nutr*., 31, 637-645.

Tempest DW, Meers JL and Brown CM, 1970: Synthesis of glutamate in Aerobacter aerogenes by a hitherto unknown route. *Biochem. J*., 117, 405-407

Terakado J, Fujihara S, Got SSS, Kuratai R, Suzuki Y, Yoshida S and Yoneyama T, 2005: Systemic effect of a brassinosteroid on root nodule formation in soybean as revealed by the application of brassinolide and brassinazaole, *Soil Sci. Plant Nutr*., 51, 389-395

Terakado J, Yoneyama T and Fujihara S, 2006: Shoot-applied polyamines suppress nodule formation in soybean (Glycine max), J. Plant Physiol., 163, 497-505

Tewari K, Suganuma T, Fujikake H, Ohtake N, Sueyoshi K, Takahashi Y and Ohyama T, 2002: Effect of deep placement of calcium cyanamide, coated urea, and urea on soybean (*Glycine max* (L.) Merr.) seed yield in relation to different inoculation methods. *Soil Sci. Plant Nutr.*, 48, 855-863

Tewari K, Minagawa R, Suganuma T, Fujikake H, Ohtake N, Sueyoshi K, Takahashi Y, Tsuchida T and Ohyama T, 2003: Effect of deep placement of slow release nitrogen fertilizers and inoculation of Bradyrhizobia on the first cropping of soybean in the field dressed with mountain soil. *Jpn. J. Soil Sci. Plant Nutr.*, 74, 183-189

Tewari K, Suganuma T, Fujikake H, Ohtake N, Sueyoshi K, Takahashi Y and Ohyama T, 2004a: Effect of deep placement of N fertilizers and different inoculation methods of bradyrhizobia on growth, N_2 fixation activity and N absorption rate of field grown soybean plants. *J. of Agronomy and Crop Science*, 190, 46-58

Tewari K, Onda M, Ito S, Yamazaki A, Fujikake H, Ohtake N, Sueyoshi K, Takahashi Y and Ohyama T, 2004b: Effect of placement of urea and coated urea on yield and quality of soybean (*Glycine max* (L.) Merr.) seed. *Soil Sci. Plant Nutr.*, 50 (8), 1245-1254

Tewari K, Onda M, Ito S, Yamazaki A, Fujikake H, Ohtake N, Sueyoshi K, Takahashi Y and Ohyama, T., 2005: ^{15}N analysis of promotive effect of deep placement of slow release N fertilizers on growth and seed yield of soybean, *Soil Sci. Plant Nutr.*, 51, 501-512

Tewari K, Onda M, Ito S, Yamazaki A, Fujikake H, Ohtake N, Sueyoshi K, Takahashi Y, Nagumo Y, Tsuchida T and Ohyama T, 2006a: Comparison of the depth of placement of lime nitrogen on growth, N_2 fixation activity, seed yield and quality of soybean (*Glycine max* (L.) Merr.) plants, *Soil Sci. Plant Nutr.*, 52, 453-463

Tewari K, Onda M, Ito S, Yamazaki A, Fujikake H, Ohtake N, Sueyoshi K, Takahashi Y, Nagumo Y, Tsuchida T and Ohyama T, 2006b: Effect of deep placement of slow release fertilizer (lime nitrogen) applied at different rates on growth, N_2 fixation and yield of soybean (*Glycine max* [L.] Merr.), *J. of Agronomy and Crop Science*, 192, 417-426.

Tewari K, Sato T, Abiko M, Ohtake N, Sueyoshi K, Takahashi Y, Nagumo Y, Tutida T and Ohyama T, 2007: Analysis of the nitrogen nutrition of soybean plants with deep placement of coated urea and lime nitrogen. , *Soil Sci. Plant Nutr.*, 53, 772-781

Vadez V and Sinclair TR, 2000: Ureide degradation pathways in intact soybean leaves. *J. Exp. Bot.*, 51, 1459-1465

Van Noorden GE, Ross JJ, Reid JB, Rolfe BG and Mathesius U, 2006: Defective long-distance auxin transport regulation in the Medicago truncatula super numeric nodules mutant[1[w]]. *Plant Physiol*., 140, 1494-1506

Veen H, 1983: Silver thiosulphate: an experimental tool in plant science. *Scientia Hort.*, 20, 211-224

Vessey JK, Walsh KB, and Layzell DB 1988: Can a limitation in phloem supply to nodules account for the inhibitory effect of nitrate on nitrogenase activity in soybean ? *Physiol. Plant.*, 74, 137-146

Vessey JK, Waterer J. 1992: In search of the mechanism of nitrate inhibition of nitrogenase activity in legume nodues: Recent development. *Physiologia Plantarum*, 84, 171-176.

Vuong TD, Nickell CD and Harper JE, 1996: Genetic and allelism analyses of hypernodulation soybean mutants from two gentetic backgrounds, *Crop Sci*., 36, 1153-1158

Warren CR and Adams MA, 2000: Capillary electrophoresis for the determination of major amino acids and sugars in foliage: application to the nitrogen nutrition of sclerophyllous species. *J. Exp. Bot.*, 51,1147-1157

Waters JK et al. 1998: Alanine, not ammonia is excreated from N_2-fixing soybean nodule bacteroids. *Proc. Natl. Acad. Sci. USA*. 95, 12038-12042

Waters JK and Emerich, DW. 2000: Transport of metabolites to and from symbiosomes and bacteroids, In: *Biology and Biochemistry of Nitrogen Fixation*. Elseveir Science Publishers, 549-558

White J, Prell J, James EK and Poole P, 2007: Nutrient sharing between symbionts. *Plant Physiol*., 144, 604-614

Wolk CP, Thomas J and Shaffer PW, 1976: Pathways of nitrogen metabolism after fixation of ^{13}N-labelled nitrogen gas by cyanobacterium, *Anabaena cylindrica*, *J. Boil. Chem*., 256, 5027-5034

Wu S and Harper JE 1991: Dinitrogen fixation potential and yield of hypernodulating soybean mutants: a field evaluation. *Crop Sci.*, 31, 1233-1240

Yamagata M, Houchi H and Yoneyama T, 1987: Partitioning and utilization of photosynthate produced at different growth stages after anthesis in soybean (*Glycine max* L. Merr.) : Analysis by long-term ^{13}C-labelling experiments, *J. Exp. Bot.*, 38, 1247-1259

Yanagisawa K, Ohyama T, Kumazawa K, 1986: Analysis of C, N accumulation in soybean seeds by $^{13}CO_2$, $^{15}N_2$ fixation and $^{15}NO_3^-$ absorption in different growth stages. *Jpn J. Soil Sci. Plant Nutr*., 57, 371-376

Yashima H, Fujikake H, Sato T, Tewari K, Ohtake N, Sueyoshi K and Takuji Ohyama, 2003: Systemic and local effects of long term application of nitrate on nodule growth and N_2 fixation in soybean (*Glycine max* (L.) Merr.). *Soil Sci. Plant Nutr.,* 49, 825-834

Yashima H, Fujikake H, Yamazaki A, Ito S, Sato T., Tewari K, Ohtake N, Sueyoshi K, Takahashi Y and Takuji Ohyama, 2005: Long-term effect of nitrate application from lower part of roots on nodulation and N_2 fixation in upper part of roots of soybean (*Glycine max* (L.) Merr.) in two-layered pot experiment. *Soil Sci. Plant Nutr.,* 51, 981-990

INDEX

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y